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Founded by K. YAMAGIWA and Continued by M. NAGAYO

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CONTENTS　目　次

Tanaka, T., and Kanô, K.: Cytological Studies of Tumors. XIII. Structural Feature and Growth of the Ascites Hepatoma of the Rat 1

田中達也，加納恭子： 腫瘍の細胞学的研究　XIII. シロネズミ腹水肝癌の構成並にその増殖 (抄録) 8

Yosida, T. H.: Tetraploi dChromosome Constitution Characteristic to the Tumor Cells of the Takeda Sarcoma 9

吉田俊秀： 武田肉腫細胞の核学的特異性 (摘要) 15

Makino, S., and Kanehisa, T.: Some Aspects of Melanotic Tumor occuring in *Drosophila Virilis* 17

牧野佐二郎，金久武晴： クロショウジョウバエにおけるメラニン性腫瘍 (摘要) 22

Mori, K.: Effect of Liver Feeding on the Production of Liver Cancer by 2-Acetylaminofluorene 23

森　和雄： 2-Acetylaminofluoreneによる肝癌生成に及ぼす肝臓飼与の影響(要旨) 28

Mori, K., Ichii, S., and Nagase, K.: Catalase and Uricase Studies on Rats during 2-Acetylaminofluorene Carcinogenesis.....29

森　和雄，一井昭五，永瀬金一郎： 2-Acetylaminofluorene 肝癌生成過程におけるカタラーゼ並にユリカーゼについて (要旨).....32

Ichii, S., Mori, K., and Ohashi, M.: Phosphatase and Esterase of Serum and Liver of Rats fed Hepatic Carcinogens 33

一井昭五，森　和雄，大橋望彦： 肝癌生成過程における白鼠の血清並に肝の Phosphatase 並に Esterase について (要旨) 39

Haruno, K.: Asparaginase Activity in Tissues of the Rats fed with Carcinogens, p-Dimethylaminoazobenzene and 2-Acetylaminofluorene 41

春野勝彦： 発癌物質経口投与ネズミの臓器アスパラギナーゼについて (要旨) 50

Kishi, S., and Haruno, K.: Enzymatic Desamination of Nucleic Acids in Liver of Rats fed on p-Dimethylaminoazobenzene 51

岸　三二，春野勝彦： アゾ色素投与白鼠肝の核酸脱アミノ酵素について (要旨) 58

Kishi, S., Haruno, K., and Asano, B.: Liver Desaminase Action on Halogenated Fatty Acid Amides in Rats fed on Hepatic Carcinogens 59

岸　三二，春野勝彦，浅野文一： 肝癌生成物質投与ダイコクネズミ肝のハロゲン化脂肪酸アミドの脱アミノ酵素について (要旨) 66

Nakahara, W., and Fukuoka, F.: Dialyzable Form of Toxohormone. A Sixth Study on Toxohormone, a Characteristic Toxic Substance Produced by Cancer Tissue.....67

中原和郎，福岡文子： トキソホルモンの可透析型 (要旨) 75

P. T. O.

Nakahara, W., and Fukuoka, F.: Biosynthesis of Toxohormone. A Seventh Study on Toxohormone, a Characteristic Toxic Substance Produced by Cancer Tissue 77

中原和郎，福岡文子： トキソホルモンの生合成 (要旨) 85

[GANN, Vol. 45: March, 1954]

CYTOLOGICAL STUDIES OF TUMORS, XIII. STRUCTURAL FEATURE AND GROWTH OF THE ASCITES HEPATOMA OF THE RAT*

(With Plate 1)

TATSUYA TANAKA and KYOKO KANÔ

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In recent years the authors, under the direction of Professor Makino, have been studying the karyological changes taking place in ordinary tissue cells during carcinogenesis. For this purpose, the senior author produced rat hepatoma with the administration of o-Aminoazotoluene and p-Dimethylaminoazobenzene (Tanaka 1952). Further the authors have attempted to transfer the rat hepatoma into a fluid form, and finally succeeded in producing an ascites hepatoma growing in the peritoneal cavity of rats (Tanaka and Kanô 1952). In the present paper, the authors propose to report on the nature and structure of the ascites hepatoma of rats thus established, with special regard to a symbiotic association between two types of cells forming the island.

Here, it is the authors' pleasant duty to express their deep gratitude to Professor Dr. Sajiro Makino for his excellent guidance and for going through the manuscript for publication. Further thanks are due to Mr. Hiroshi Nakahara for his kind aid in making photographs and for some technical advices.

MATERIAL AND METHODS

The wistar albinos (*Rattus norvegicus*) inbred purely in our colony were used as host for the transplantation of the ascites hepatoma. In the ascites hepatoma the neoplastic exudates can be obtained simply by puncturing the abdominal cavity with glass capillaries as is done in other ascites tumors.

Since employment of Giemsa's technique causes artificial cohesion and irregular deformation of hepatoma islands, microscopical observations were mainly performed with the smear preparations according to the acetic gentian violet and acetic dahlia technique (Tanaka and Kanô 1952). The preparations for the daily observations were made beginning with the second day after transplantation of the ascites hepatoma.

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* Contribution No. 298 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Aided by a grant from the Scientific Research Fund of the Ministry of Education.

Observations

1. General feature of the hepatoma islands observed in the neoplastic exudates

The examination of smear preparations made from well-grown neoplastic exudates showed a large number of hepatoma islands which are suspended in the peritoneal fluid (Figs. 1, 12). Structurally, the hepatoma island is an aggregation of a certain number of hepatoma cells (cancerous hepatic cells) of varying sizes and shapes, in association with some endothelial cells. That is to say, a hepatoma island consists of two cellular elements, hepatoma cells and endothelial cells which are different in origin and nature. A few endothelial cells occur adhering the surface of the hepatoma cell mass (fig. 2). The hepatoma cells are hepatic parenchymal in origin. The cells of the hepatoma island vary in size in a considerable degree, but they are very prominent due to their being of larger size than any other cells observable in the nontumorous or inflammatory peritoneal fluid of the rat. Their distinguishing feature is to contain a spherical or oval nucleus, located in the central part of the cytoplasm. The nucleus shows two or three nucleoli-like bodies, small in size but distinct in appearance. The nucleoli-like bodies of the hepatoma cells were more condensed in form than those of the endothelial cells. Binucleate cells are not uncommon (Fig. 3). There are a number of granules distributed throughout the cytoplasm. Many of them were found to lie in the transitional region between the ectoplasm and endoplasm, showing an active movement. These bodies were various in shape and easily distinguishable from the mitochondria by their rough contour, and by their affinity for neutral red or trypan blue.

The endothelial cells are characterized by a small amount of cytoplasm carrying a kidney- or horseshoe-shaped nucleus. The nucleus was colorless being not granulated in a living state, and it contained a larger amount of chromatin material than the parenchymatous one in the stained preparation. The nucleoli are variable in their position, size and shape. The cytoplasm is dense and homogeneous, but granular around the nucleus in living preparations. In the stained preparations the homogeneous ectoplasm stained very faintly and it was well distinguishable from the endoplasm, which was granulated and deeply stained (Fig. 4). The ectoplasm transforms sometimes into an undulating fan-like form and displays a characteristic amoeboid movement. The ectoplasm of the endothelial cell adheres the one side of the hepatoma cell (Figs. 5-6). Sometimes, the ectoplasm extends to surround the whole surface of hepatoma cell body.

Injection of Indian-ink solution into the peritoneal cavity of the tumor-bearing animal proved an appreciable phagocytic activity of the endothelial

cells. The cytoplasmic granules are less in number in the endothelial cell than in the hepatoma cell. By these characteristic features, the endothelial cells are clearly distinguishable from the hepatoma cells.

As a whole, the hepatoma island is of a spherical or oval shape in a well matured neoplastic exudate. The shape of an island, however, varies considerably at different stages after transplantation of the ascites hepatoma. In the early to middle parts of the life span of tumor-bearing rats the island appears in a grape- or cord-like form. Towards the latter part of the animal's life, they increase gradually in volume, assuming a spherical form. Probably this is due to surface tension. The mitotic figures were observed along the peripheral region of the island.

Observations through successive transplant generations have revealed that the hepatoma island is produced in two general ways; the one is due to an direct separation from the mother hepatoma island, and the other is through a disintegration of the hepatoma island, as to be mentioned below.

2. Occurrence of the tumor cells freely being suspended in the peritoneal fluid

In addition to the hepatoma islands as described above, there are found a number of free cells suspended in the peritoneal fluid of the ascites hepatoma rat. They are abundant in the early part of the life span of the tumor-bearing rat, and decrease gradually towards the latter part. Two general types of free cells are discernible. The predominant type is represented by a remarkably large spherical cell containing a number of granules in its cytoplasm. The nucleus is spherical or oval. In its general morphological characteristics, such a cell resembles very much the typical parenchymal cell (Fig. 7).

The cell of the second category is larger in size than the former, being much elongated. It has a dense cytoplasm showing an undulating margin. It is characterized by an amoeboid movement with pseudopodia. It shows a number of cytoplasmic granules which are distinguishable from those of the former type on account of their highly refractive nature. From these characteristic features, it is considered highly probable that the cell of this type is a derivative of the endothelial cell. But, it is difficult to say whether it is derivative from the endothelial cell occurring in the liver (Figs. 8-9).

Mitotic figures of these free cells are observable rather frequently, but the cells which undergo the regular mitosis are few in number. Most cells showed abnormality in the process of cell division. Interestingly, the parenchymal cells tend to show the degenerative features rather more frequently than the endothelial cells. It seems to the authors that the parenchymal cells display their activity when in association with the endothelial cells. This is presumable from the fact that the component elements of the ascites hepatoma are essentially the hepatoma islands which always occur in the symbiotic association of the

hepatoma cells and the endothelial cells.

3. Growth and structural changes of the ascites hepatoma in the successive transplantations

After the inoculation the ascites hepatoma grows rapidly in the peritoneal cavity of the new host, and causes the death of the tumor-bearing animal in ten days on the average. The behavior of tumor cells was followed in a transplant generation of the ascites hepatoma, as described below:

The neoplastic exudates inoculated in the new host contain hepatoma islands of various sizes ranging from small globes to large cell masses. Following the transplantation a great many of the hepatoma islands introduced into the peritoneal cavity of the new host undergo disintegration. The majority of disintegrating islands are those of large size, while small hepatoma islands seem to remain without undergoing degeneration. In general, disintegration of the hepatoma island seems to be the result of vacuolization of hepatoma cells (Fig. 10). The vacuoles are well demonstrable when stained with neutral red. The vacuoles appear first in any part of the cytoplasm. They increase in size and number with time, and finally occupy the whole cytoplasmic area. By this time, the cells are stained very deeply with neutral red. Meanwhile, blister-like processes of varying lengths protrude from the surface of the hepatoma cells. In the next stage the disintegration of the island occurs, resulting in the production of freely suspended cells along with the formation of small-sized hepatoma islands containing a few cells (Fig. 11).

On the 1st and 2nd day after transplantation, there occur in the ascites many small islands, consisting of several tumor cells, together with a number of free cells. The mitotic figures of hepatoma cells begin to appear at about 30 hours after transplantation showing a gradual increase with time.

About three days after transplantation, the hepatoma cells lying in the peripheral part proliferate actively. The formation of the hepatoma island seems to be largely influenced by the mitotic activity of hepatoma cells and endothelial cells. The hepatoma islands assume various shapes, showing sphere-, grape-, or cord-like appearance according to the multiplication of cells. Towards the middle part of the life span of the tumor-bearing animal, the cells undergoing mitosis in the island are highest in frequency. By that time, the separation of the large island into several small ones has taken place very actively. As a result the number of the hepatoma island remarkably increase. Thus, a "pure culture" condition of the ascites hepatoma is attained in the peritoneal fluid (Fig. 12).

Towards the latter part of the tumor-animal's life the mitotic cells in the hepatoma islands decrease in number. With the decrease of the mitotic cells, the number of the spherical islands increases. At this stage all the islands have

a covering of the endothelial cells, assuming a spherical form. The morphological differentiation of the endothelial cell from the hepatoma cell is very marked.

With these changes the tumor ascites shows a gradual accumulation. Nearing the last day of the host animal's life, dividing hepatoma cells decrease in number. The disintegrating hepatoma islands are also remarkable. By that time the accumulation of the tumor ascites attains an enormous amount with a great expansion of the abdomen of the tumor-bearing animal. Finally the malignant growth of the tumor leads to the death of the host animal. By intraperitoneal transmission of these neoplastic exudates into a new host, the hepatoma islands begin their cycle changes in transplantation.

In the ascites hepatoma, the whole life span of the tumor-bearing animal, ranging from the first day of transplantation of the tumor ascites to the death of the host animal, is rather short, as is also the case in the MTK-sarcomas (Tanaka and Kanô 1951), as well as in the Yoshida sarcoma (Yoshida 1949). Most of the tumor-bearing rats died on the 10th to 12th day after transplantation, being on the 10th day on the average. An exceptional tumor-animal died on the 22nd day after transplantation.

4. Rate of mitosis in a transplant generation

The mitotic rate was observed in the ascites hepatoma in the same manner as was done in the Yoshida sarcoma and MTK-sarcomas. Namely, two thousand tumor cells were observed every day through the whole transplant generation, and daily frequencies of all dividing cells showing late prophase, metaphase, anaphase and telophase, were calculated in percentage. The results are given

Table 1. Daily frequency of mitotic cells in an ascites hepatoma rat. The percentage of dividing cells was calculated on the basis of 2000 cells per day in the observation through a transplant generation.

Days after transplantation	1	2	3	4	5	6	7	8	9	10
% of dividing cells	1.6	2.4	3.2	4.2	4.5	3.9	2.4	1.6	0.8	0.6

in Table 1. Generally speaking, the number of dividing cells strikingly increases during the early part of transplant generation, showing the highest frequency during the middle part. Then, the mitotic rate decreases gradually towards the latter part of the tumor-bearing animal's life.

Remarks

Several attempts have been made to transfer a malignant tumor into a fluid form (Hesse 1927, Loewenthal 1932, Goldie and Felix 1950, Klein and Klein 1951, Usubuchi 1952, Takeda 1952). It has been found that the following mouse

tumors: S-37, malignant lymphoma, S-180, melanoma, Ehrlich adenocarcinoma and Krebs-2, can be freely grown in the peritoneal cavity of mice by successive transmissions. In these ascites tumors, the individual tumor cells are usually able to maintain themselves in an isolated form, or are provided with some nutritive material from the ascites fluid in an isolated state. In striking contrast to the above-mentioned tumors, the ascites hepatoma here concerned can be grown only under a symbiotic association between the hepatoma cells and the endothelial cells forming the hepatoma island. Therefore, each hepatoma island is to be regarded as an autonomous unit in the ascites hepatoma of the rat, so far as the present material is concerned.

The hepatoma island consists of two cellular elements, hepatoma cells and endothelial cells differing both in origin and in nature. The endothelial cells always occur adhering to the hepatoma cell mass. The separation into small islands from a large mother island always occurs in an associated condition of the two types of cells, as demonstrated by Tanaka (1953). Also, it has been observed that the hepatoma cells can divide and grow in a close association with the endothelial cells in the ascites hepatoma (Tanaka 1953).

According to Holtfreter (1948), the gathering of free cells depends on chance, but their subsequent cohesion to form clusters is governed by certain physicochemical conditions of their surface. The concepts of 'contiguity effects' and 'tissue affinity' proposed by Holtfreter (1939) are interesting and important in considering the intimate association of two cell elements occurring in the hepatoma island here concerned.

Summary

The structural features and growth of the ascites hepatoma of the rat were observed throughout the whole life span of the tumor-bearing animals. The structural unit of the ascites hepatoma is a hepatoma island consisting of two cellular elements, cancerous hepatic cells and endothelial cells, which are in a symbiotic association. The hepatoma cells of the ascites hepatoma show their regular mitotic activity in the presence of the endothelial cells. Suggestion was made that the hepatoma island can accomplish its characteristic growth and function under the symbiotic association of the hepatoma cells and endothelial cells.

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Explanation of Plate

Fig. 1. Hepatoma island, containg dividing hepatoma cells, from the neoplastic exudates on the 4th day after transplantation, × *600*. Fig. 2. Hepatoma island showing a remarkable endothelial cell layer, × *600*. Figs. 3–4. Small hepatoma islands, showing the endothelial cells adhering the surface of the hepatoma cells, × *1200*. Figs. 5–6. Mitotic figures of hepatoma cells in the small islands, × *1200*. Figs. 7–9. Tumor cells freely suspending in the neoplastic exudates, × *800*. 7, free hepatoma cells and an elongated endothelial cell. 8–9, free endothelial cells showing the amoeboid movement with pseudopodia. Fig. 10. Disintegrating hepatoma island from the neoplastic exudates at about 24 hours after transplantation, × *600*. Fig. 11. Small hepatoma island and free cells produced by the disintegration of hepatoma island, × *600*. Fig. 12. Hepatoma islands showing a pure culture condition, from the neoplastic exudates on the 5th day transplantation, × *400*. Figs. 1, 4, 5, 6 and 12, from acetic gentian violet preparations. Figs. 2–3 and 7–11, from the living preparations, taken with the aid of an Olympus phase-contrast microscope).

抄　録

腫瘍の細胞学的研究 XIII. シロネズミ腹水肝癌の構造と増殖

田中達也・加納恭子

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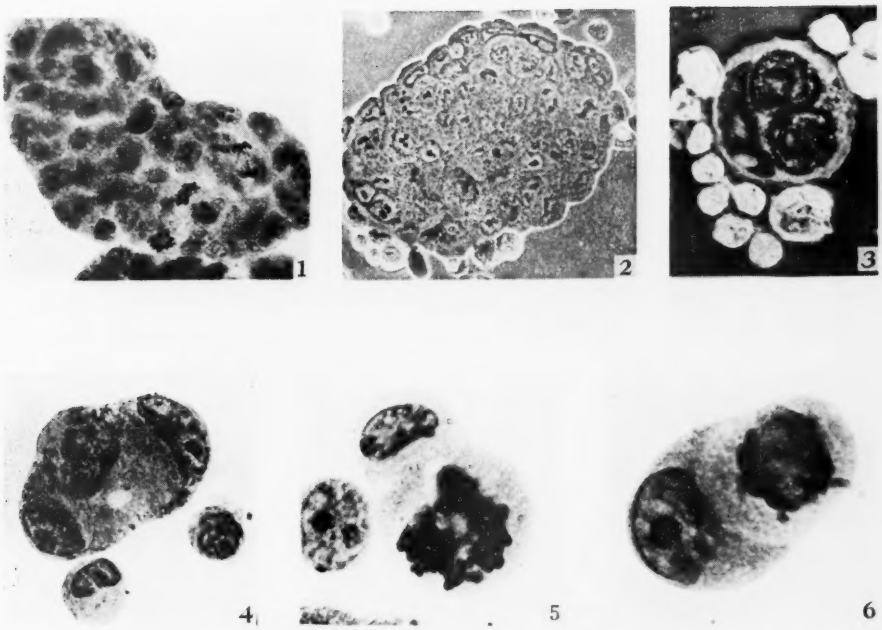
著者等は過去数年来発癌過程における組織細胞内に生ずる核学的変化を研究中であるが，この目的のために著者の一人，田中 (1952) はシロネズミ (*Rattus norvegicus*) にアゾ色素 (o-Aminoazotoluene, および p-Dimethylaminoazobenzene) を投与し，その発癌過程における染色体の変化を細胞学的に調査した。しかしながら，ここに得られた結果は，多くの実験動物に観察された集成的結果であり，肝癌細胞の詳細な核型の調査は，当然1系統の肝癌系について連続的に研究を進める必要がある。従つてもしアゾ色素投与によつて生じた肝癌を腹水系に置換することができれば，移植操作，ならびに染色体の連続調査を極めて容易に実施することができるので，アゾ肝癌の腹水系への置換を試みた。この結果新に2系統の腹水肝癌系の確立を認めた (田中，加納 1952)。この研究は，かくして得られた腹水肝癌の一般構造，並にその増殖過程の観察結果である。

腹水肝癌は構造的にみて，細胞起原を異にする2種の細胞，すなわち肝癌細胞と内皮細胞とによつて構成された1箇の細胞集団である。内皮細胞は，肝癌細胞の周辺部を囲繞して存在し，その細胞形態は原形質のアメーバー状運動，曇粒貪食能，ならびに位相差顕微鏡下における高度の屈折率等により明瞭に識別することができる。肝癌島の形態は，主として球状であるが，移植後の腫瘍成長の度合によつて球状，索状あるいは葡萄状など種々の形態的変異が観察される。肝癌島の増加は，移植後に生ずる島の分解遊離，及び母肝癌島の自然的切断によつて生ずる。

腫瘍腹水の中には上記肝癌島の外，腹水中に単独で浮遊する自由遊離細胞が観察される。かかる自由遊離細胞は肝癌島の分解遊離，あるいは母肝癌島よりの遊離脱落によつて生ずるものであつて，移植後 2~3 日において特に顕著に観察することができる。自由遊離細胞の分裂像もまたこの期間にしばしば観察されるが，その大部分は分裂の途中において変性退行の傾向を示す。

以上の観察結果に基いて，腹水肝癌は，その構成細胞である肝癌細胞と内皮細胞との密接なる結合状態において，正常の機能を営むものであり，かつまた肝癌島内の細胞相互の結合は，細胞表面の物理化学的組織親和性に基因するものと考えられる。(文部省科学研究費による)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[GANN, Vol. 45; March, 1954]

TETRAPLOID CHROMOSOME CONSTITUTION CHARACTERISTIC OF THE TUMOR CELLS OF THE TAKEDA SARCOMA* **

(With Plate II-IV)

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Karyological investigations of the ascites tumors of rats continuously carried out recently by Makino (1951, 1952 a, b), Makino and Yosida (1951), Makino and Kanô (1951, 1953), Yosida (1952), Tanaka and Kanô (1951) and Makino and Tanaka (1953 a, b) in the Yoshida sarcoma, MTK-sarcoma I and II, and the Hirosaki sarcoma, have made it clear that, in each of the four kinds of ascites tumors, three of which were experimentally induced and one of which was spontaneous in origin, there is present a strain (or strains) of tumor cells, having a peculiarly characteristic chromosome constitution, that multiply with almost regular mitotic behavior. The individuality of chromosomes in these strain-cells has remained unchanged through successive transplant generations from rat to rat. The growth of the tumor is primarily the result of proliferation of these tumor strain-cells. In every case, the tumor strain-cells show a well-balanced subdiploid complex of chromosomes, 40 or thereabouts in number. The chromosome complex consists of two distinct groups, namely a group of rod-shaped elements and a group of V- and J-shaped ones. The four kinds of ascites tumors are characterized by different numbers of the rod-, V- and J-shaped chromosomes. Evidence presented is of great significance in connection with the mechanism of growth of malignant tumors, as well as with the question of how the tumor cells arose or were transformed from ordinary tissue cells.

The present study has been made in an attempt to obtain evidence on the possible occurrence of similar phenomena in the Takeda sarcoma which is a sort of ascites tumor in rats. According to Professor K. Takeda, this tumor developed ordinarily from the transplantation of a spontaneous solid tumor of unknown origin found in a white rat in the Pathological Laboratory, Hokkaido University. The rats receiving a transplantation of this tumor in their abdominal cavities died after ten days or more. By peritoneal transplantations successive transmissions have been continued from rat to rat. Though not completely

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analyzed, the essential nature of this sarcoma was said to be nearly similar to that of the Yoshida sarcoma as well as to that of the MTK-sarcomas.

The author takes much pleasure in expressing his sincere gratitude to Professor Sajiro Makino of Hokkaido University for kind revision of the manuscript for publication. Further thanks should be extended to Dr. Kan Oguma, the director of the National Institute of Genetics, for his keen interest and for valuable advice. The author is also obliged to Professor Katsuo Takeda who placed the present material at his disposal.

The study was carried out by the use of smear preparation of the ascites tumor stained with aceto-orcein. The technique was described in detail in the former paper (Makino and Yosida 1951).

Results of observations

1. Frequency of tetraploid cells: Upon observations of the slide the author was surprised to find that the Takeda sarcoma is represented by apparently large sized tumor cells. Close observations of the metaphase chromosomes made it clear that those tumor cells are characterized by having a tetraploid (or subtetraploid) chromosome complement. The frequency of the tetraploid cells was observed in the material coming from two different tumor-bearing rats. The study was made based on the following three types of cells; diploid ($\pm 2n$), tetraploid ($\pm 4n$), and hyperploid ($>\pm 4n$). In a specimen (A) which died on the seventh day after transplantation of the tumor, 127 tumor cells at metaphase were observed for this purpose. Of the cells studied, 106 cells (80 %) were tetraploid, 14 cells (11.0 %) showed diploid and the remaining 7 cells (5.5 %) were hyperploid in their chromosome complex. In another specimen (B) which showed nine days' life span, the relative frequency of the di-, tetra- and hyperploid cells were found to be 22.4, 70.5 and 6.9 per cent, respectively (Table 1). It is evident from these results that most of the tumor cells are characterized by the tetraploid complement. There is no doubt, considering the evidence observed in other ascites tumors by Makino (1952 a, b) and Makino and Kanô

Table 1. Frequencies of diploid, tetraploid and hyperploid cells occurring in the tumor.

Specimen		Dipl. ($\pm 2n$)	Tetrapl. ($\pm 4n$)	Hyperpl. ($>\pm 4n$)	Total
A	No. of cells obs.	14	106	7	127
	%	11.0	83.4	5.5	99.9
B	No. of cells obs.	29	91	9	129
	%	22.4	70.5	6.9	99.9

(1953), that these cells with tetraploid complex are the tumor strain-cells which serve as primary contributors to the growth of the tumor.

As a matter of fact, these strain-cells multiply by regular mitosis, as shown by the fact that the greater part of the mitotic cells are represented by the regularly dividing cells showing subtetraploid chromosomes (Table 2).

II. Abnormal mitoses: In the Takeda sarcoma, as in others, various types of abnormal mitoses were observed throughout a transplant generation. The following mitotic abnormalities were noticed: stickiness and coalescence of chromosomes, abnormal swelling or vacuolization of chromosomes, deformation of chromosomes into unusual forms, disintegration of chromosome spirals, non-disjunction and lagging of chromosomes, the chromosome bridges, irregular chromosome distribution at anaphase, scattering of chromosomes at metaphase, abnormal orientation of chromosomes, multinucleated cells and multipolar mitoses (Figs. 14–19). It is clear that cells undergoing abnormal mitoses or being in process of disintegration can not pursue active multiplication and die sooner or later. The cells with well-balanced subtetraploid chromosomes which multiply with almost regular mitotic behavior contribute primarily to the growth of the tumor. In the course of multiplication, part of the proliferating cells become abnormal and undergo aberrant mitotic processes owing probably to an alteration of the spindle mechanism, structural change of the chromosomes and some other causes. The tumor cells showing mitotic abnormalities are, therefore, derivatives of the tumor strain-cells.

Table 2. Frequencies of cells showing regular mitosis, aberrant and disintegrating configurations observed in the tumor strain-cells ($\doteq 4n$ cells).

Specimen		Regularly mitotic cells	Aberrant cells at metaphase	Disintegrating cells at metaphase	Total
A	No. of cells obs.	57	20	29	106
	%	53.7	18.6	27.7	99.6
B	No. of cells obs.	49	26	16	91
	%	53.8	28.5	17.5	99.8

III. Chromosome numbers of the tumor strain-cells: The chromosome count was made in some metaphase plates available for study. The results are given in Table 3. The chromosome numbers show a wide range of variation between 36 and 104. Though the data here obtained are rather meagre due to the small number of the observed cells, it is still interesting to see that the cells possessing the chromosome number of 84 or thereabouts have been found in high frequency. Considering the corresponding data obtained in other ascites tumors it is certain that these cells with a subtetraploid chromosome complex play an

important role in the formation of the tumor.

Table 3. Number of the chromosomes observed in the thumor cells.

No. of chrom.	No. of cells obs.	No. of chrom.	No. of cells obs.
104	1	73	1
89	2	69	1
86	1	48	1
85	1	43	1
84	4	40	2
83	1	38	3
82	1	37	1
80	2	36	2
76	1	Total	26

IV. Chromosome constitution of the tumor strain-cells: As stated above, the tumor strain-cells are characterized by a subtetraploid complex of chromosomes fluctuating in number around 84. The chromosomes are arranged in the normal radial manner, the large ones occupying peripheral positions surrounding the smaller ones. Neither in the orientation of the chromosomes nor in the spindle formation is there anything unusual. In these strain-cells the chromosomes were analyzed by comparing them to those of the Yoshida sarcoma, MTK-sarcoma I and II, and Hirosaki sarcoma.

Upon a glance at the metaphase plate, the chromosome complex of these tumor cells is seen to be very motley because of the existence of many J- and V-shaped elements of various shapes and sizes. Among them, the presence of a prominent J-shaped element is particularly remarkable, on account of its conspicuously large size. The occurrence of such a J-shaped element is common in many tumor cells (Figs. 1–5, 10–13, and 20–23). Occasionally, the large J-shaped element displays a tripartite structure as seen in Figes. 3 and 23. This fact suggests the possibility that it originated by association of three rod-shaped elements in a linear series. The large V-shaped chromosome which shows a similar configuration to that regularly found in the Yoshida sarcoma cells is also common in this tumor, numbering one in most cases and rarely two (Figs. 1–6, 10–13 and 20–23). Though not completely analyzed, there are 19 to 24 small V-shaped chromosomes in addition to the above outstanding elements and a certain number of red-shaped elements. More detailed research is needed in future for the completion of the morphological analysis of the chromosomes of the tumor cells.

Concluding Remarks

Makino (I952 a, b) has found that the tumors strain-cells of the Yoshida sarcoma are characterized by having about 40 chromosomes consisting of two groups, namely 22 to 24 rod-shaped elements in one group, and 16–18 V- or J-shaped elements in the other, including a prominent V-shaped element of large size. Recently Makino and Kanô (1953) studied the chromosomes of tumor strain-cells in the MTK-sarcoma I and II, and Hirosaki sarcoma. A major conclusion to be drawn from their study was that in each of the tumors there is present a strain (or strains) of tumor cells having characteristic chromosome constitutions specific to the kind of tumor, and remaining unaltered in chromosome individuality through successive transplant generations. The tumor strain-cells show a well-balanced subdiploid complex of chromosomes, 40 or thereabouts in number. The chromosome complex consists of a group of rod-shaped chromosomes and another of V- and J-shaped ones. The three kinds of ascites tumors are characterized by different numbers of the rod-, V- and J-shaped chromosomes. It is evident by referring to the results of the present study that the situation in the Takeda sarcoma is highly different from that in the other tumors as mentioned above, respectively the chromosome constitution of the tumor cells. The chromosome constitution of tumor strain-cells of the Takeda sarcoma is unique because of its tetraploid nature; the strain-cells are characterized by having a sub-tetraploid chromosome complex, fluctuating in number around 84, and including a certain number of V-, J- and rod-shaped elements. It is noticeable that among the composing elements, a large tri-partite J-shaped chromosome and the large V-shaped element, one or rarely two in number, are very prominent due to their remarkable size and shape.

Levan and Houschka (1952) reported that the Ehrlich ascites tumor in mice is represented by tumor-strain cells having a tetraploid complex of chromosomes. The present author (1954 a, b) established, in the Takizawa quinone carcinoma and the Ehrlich ascites tumor of mice, the occurrence of the tumor strain-cells characterized by the tetraploid complex of chromosomes. The tumor cells of these mice tumors are characterized by the presence of V-shaped chromosomes of medium size, numbering one or rarely two, in addition to rod-shaped elements.

As an explanation for the origin of the particular chromosome complexes found in the tumor strain-cells of the four kinds of ascites tumors of rats, Makino (1952 b) and Makino and Kanô (1953) have postulated the occurrence of chromosome mutations during the transformation of an ordinary tissue cell (or cells) into a tumor cell (or cells). They stated that, perhaps at some stage during experimentation with the carcinogenic agent, or in the life history of an

individual, a mutational change took place in the chromosomes of an ordinary tissue cell (or cells). Probably, the process of mutation was such that it has not essentially disarranged the elements of cell metabolism or of mitotic activity, but has enabled the mutated cells to grow autonomously. These cells multiplied to produce a strain (or strains) of tumor cells in which the altered chromosome constitution and the capacity for autonomous growth were preserved. It seems likely that the view expressed by Makino (1952) and Makino and Kanô (1953) is applicable also for explanation of the transformation of the normal non-tumor cells into the malignant cells of the Takeda sarcoma which are characterized by a tetraploid complex of chromosomes.

Summary

The present paper describes the results of the karyological study of the tumor cells of the Takeda sarcoma in rats. It was found that there is present a strain (or strains) of tumor cells hoving a subtetraploid complex of chromosomes, and remaining unaltered in chromosome individuality through succesive transplant generations. The growth of the tumor is due primarily to the proliferation of these strain cells. The chromosome complex of the strain-cell consists of two distinct groups, namely a group of rod-shaped chromosomes and a group of V-, and J-shaped ones of varying sizes. Among the two-armed chromosomes, a large J-shaped chromosome, tripartite in structure, and a large V-shaped element, one or rarely two in number, are very prominent due to their outstanding size.

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Explanation of Plate IV

Photomicrographs of tumor cells. Figs. 14–16, tumor cells showing multipolar division, 14, tetraploid metaphase. 15, tripolar anaphase. 16, tripolar anaphase, showing chromatin bridges. 17, bipolar anaphase, showing chromatin bridges. Fig. 18, deformation of chromosomes into round form. Fig. 19, scattering of chromosomes in the metaphase plate. Figs. 20–23, metaphase plates showing subtetraploid chromosome complexes. Fig. 21 from Fig. 4. Fig. 22 from Fig. 5. Fig. 23 from Fig. 3. (Large J-shaped chromosomes are indicated by arrows). (Figs. 14–19 ; ×600. 20, 22, 23 ; ×1000. 21 ; ×800).

摘　　要

武田肉腫細胞の核学的特異性

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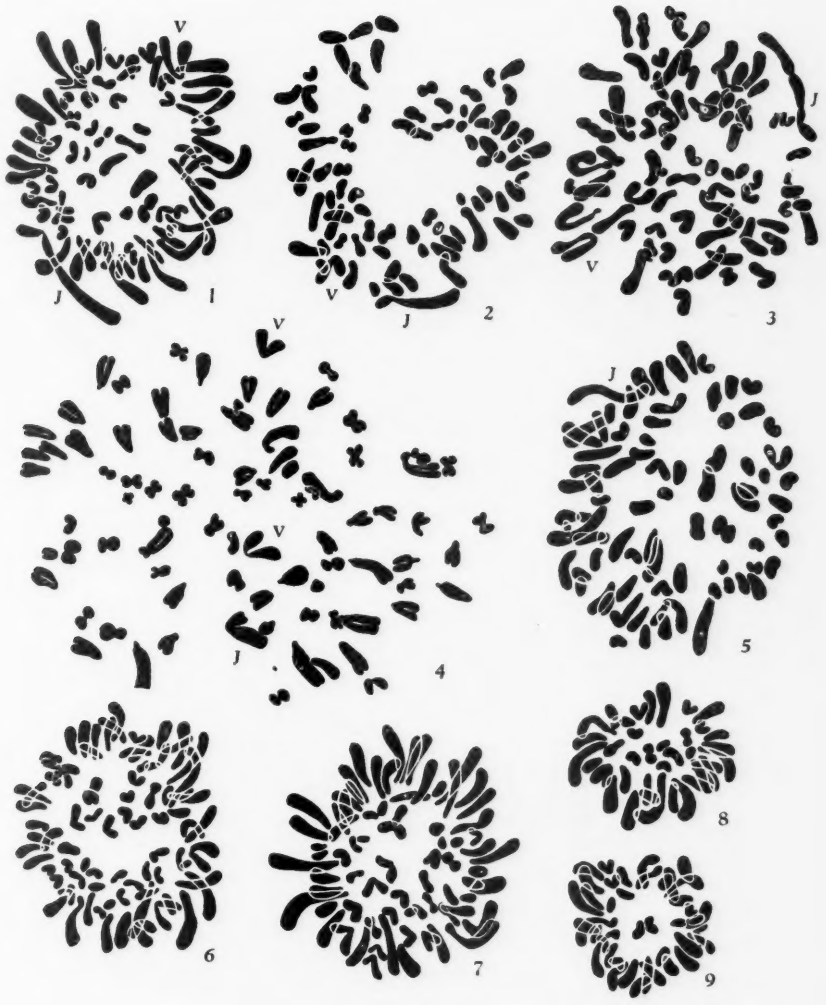
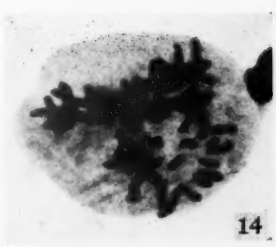
武田肉腫とはラットにおける腹水性腫瘍の一系統である。この腫瘍は四倍性の細胞が多く，観察された (A) 個体では 83.4%, (B) 個体では 70.5% が四倍性であつた。これらの結果から，武田肉腫の種族細胞は四倍性であると考えられる。四倍性細胞の染色体数を詳細に調べてみると，ラットの染色体数の倍数である 84 前後のものが最も多い。次に各染色体の形態を詳細に調べてみたところ，通常の棒状染色体の外に，小型の V あるいは J 字形染色体が 19 から 24 個，大型の V 字形染色体が 1 個あるいは 2 個観察され，しかも核型分析をされた多くの細胞には大型の J 字形染色体が 1 個観察された。大型の J 字形染色体は正常マウスの体細胞や他の二倍性腹水腫瘍 (例えば吉田肉腫，MTK 肉腫等) には観察されない，武田肉腫の種族細胞における核学的特異性であると考えられた。



Camera-lucida drawing of tumor cells, ×1000.

Figs. 1–7, metaphase plates showing subtetraploid chromosome complex (1; 86 chroms. 2; 82 chroms. 3; 80 chroms. 4; 84 chroms. 5; 80 chroms. 6; 83 chroms. 7; 76 chroms.). Figs. 8–9, metaphase plates showing subdiploid chromosome complexes. (8; 38 chroms. 9; 40 chroms.). J=large J-shaped chroms. V=large V-shaped chroms.

Figs. 10–13, serial alignments of chromosomes in tumor cells. Fig. 10 from Fig. 1. Fig. 11 from Fig. 3. Fig. 12 from Fig. 2. Fig. 13 from Fig. 4. In each complement, one element is represented by a large J-shaped element, one or two by large V-shaped ones together with 56–65 rod-shaped elements and 19–24 small V-shaped ones.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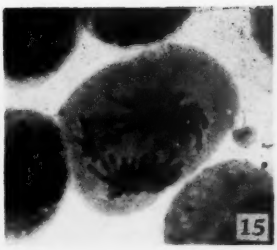
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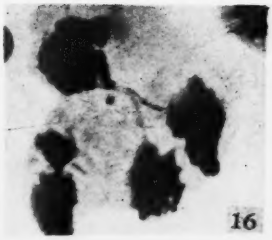
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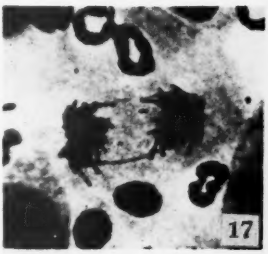
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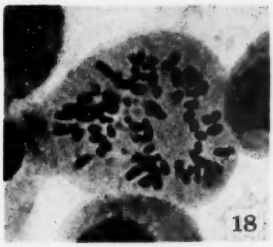
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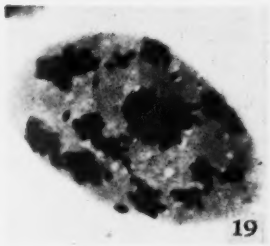
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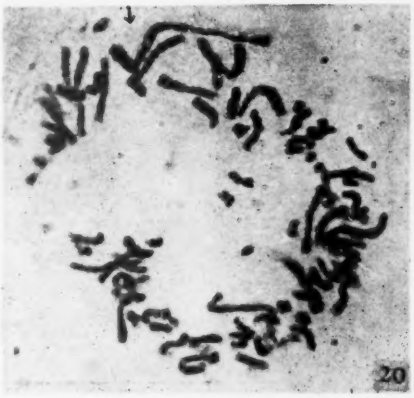
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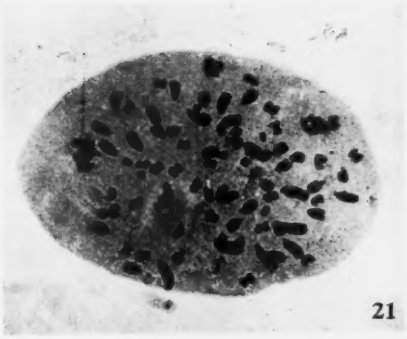
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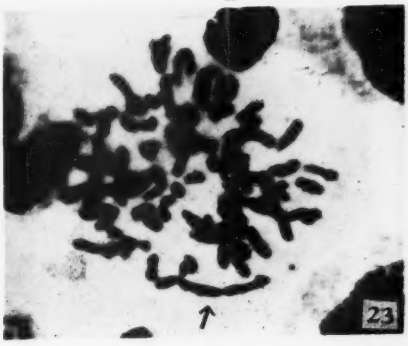
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[GANN, Vol. 45; March, 1954]

SOME ASPECTS OF THE MELANOTIC TUMOR OCCURRING IN DROSOPHILA VIRILIS*

(With Plate V)

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Because cancer represents a form of uncontrollably rapid growth occurring in an organism through a continuous series of descendant cell generations, the study of tissue growth constitutes the central problem of tumor research, and its biological significance is very great. From the broader point of view, an analysis of cancerous growth should include representatives from all kinds of living organisms. In the animal kingdom pathological study has dealt largely with neoplasms in various kinds of vertebrates, while invertebrates have been left largely unexplored in every field of cancer research. Most probably this is due to the fact that pathologists in cancer research are not generally familiar with invertebrate animals. It has been recognized that invertebrate tissues are capable of supporting cancerous growth. Obviously, they offer an opportunity to approach the study of tumors in new ways. Because of the vast background of genetic knowledge of *Drosophila*, it is not surprising that hereditary tumors have been described in this insect. Stark ('18) was the first to study such tumors in *Drosophila* using a strain discovered by Bridges. The earlier works were carried out in relation mainly to the genetic differences, while more recent studies have dealt with the effects of temperature, X-rays and some other agents. A review of the literature in this field has been published by Scharrer and Lochhead ('50).

Recently we have had an opportunity to study the tumorous growth in *Drosophila virilis* which was derived from wild stocks obtained in Sapporo, 1950. Since the appearance of these tumors resemble that of the melanotic tumors described by earlier authors (Wilson '24, Stark '37, Russell '40, Herskowitz & Burdette '51, Burdette '50, '51), they were regarded as similar melanotic tumors. They appeared with a considerably high frequency. In addition, a similar type of tumors has been discovered to occur in the same species from other strains

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which came from different localities, though not with higher incidence than in the former.

The present paper deals with the morphological and genetical studies of these tumors found in *D. virilis* Though the data obtained are somewhat fragmental, the following notes may not be entirely uncalled for.

Keen interest together with valuable advice extended by Dr. S. Fujii and Mr. M. Kawabe, Kobe University, regarding this subject is acknowledged here with cordial thanks.

Observations

The tumorous growths which occurred in *D. virilis* were recognized as melanotic specks or aggregations by reference to those described in *D. melanogaster*. They were usually external and associated with the hypodermis. They appeared mainly on the mesonotum, scutellum, pleula, and head regions (Figs. 1–9). They were irregular in shape and size, and varied in number from one to three in a single individual.

The flies from the original stock obtained in Maruyama, Sapporo City, manifested the tumor-incidence of only 3.4 per cent, while by selection and inbreeding a considerable increase of the incidence was obtained with succeeding generations. As shown in Table 1, an incidence of 44. 2 in the F_1-generation was stepped up to 91.0 per cent in the F_4-generation. But the expressivity of the tumor was observed to decrease inversely with generations.

The tumors did not appear pigmented bodies during the larval and pupal stages. They were morphologically discernible in the imagos, about two to three weeks after emergence when meal-molasses at about 25° C was used as the culture medium. The tumors seemed to be of the benign type because of their non-lethal effect upon the host, giving no evidence of malignancy.

The tumors are variable in appearance according to their developmental conditions. Examples are shown in Figures 1 to 5. Figure 1 shows a crust-like body, slightly pigmented, developing on the mesonotum, while in Figure 2 the abnormal growth appears as a pigmented black speck. Most of them disappeared with

Table 1. Results of inbreeding, showing the increase of the tumor incidence with generations.

Generation	tu		+		Total		% of	
	♀	♂	♀	♂	♀	♂	Total	tu
1 st	78	58	75	96	153	154	307	44.2
2 nd	44	37	29	32	73	69	142	58.0
3 rd	45	19	4	2	49	21	70	94.0
4 th	218	154	17	16	235	170	405	91.0

the age of the adult fly. The appearance of the tumorous growth as discrete black bodies is shown in Figures 3, 6 and 7. Frequently these bodies tend to reduce their pigmentation with age (Fig. 3, a–d). It was found in certain flies that the crust-like body developed changing into the discrete black body due to the melanotic aggregation, which was then followed by decrease in pigmentation with age until it finally disappeared (Fig. 4). Further, there were some flies showing the tumorous growth as melanotic aggregations with fat-like accumulation, yellowish white in colour (Figs. 5, 8, 9).

Some experiments on outcrossing attempted between the tumor-strain and the wild strains obtained from two different localities of Hokkaido (Higashitakasu and Otaru) have resulted in a suggestion of the maternal influence in tumor production. Further experiments were carried out with a purpose to confirm the maternal influence in crossing with wild stocks from another two localities

Table 2. Data from outcrossing.

Cultures		+ × tu						tu × +					
		+(Iz) × tu (My)			+(Ot) × tu (My)			tu (My) × + (Iz)			tu (My) × + (Ot)		
		Total	tu	% of tu	Total	tu	% of tu	Total	tu	% of tu	Total	tu	% of tu
A	♀	25	7	28.0	56	13	23.2	75	43	57.3	54	33	61.1
	♂	27	14	51.8	55	6	10.9	51	22	43.1	57	37	64.9
	T	52	21	40.3	111	19	17.1	126	65	51.5	111	70	63.0
B	♀	34	14	41.1	61	20	32.7	65	27	41.5	70	38	54.2
	♂	33	13	39.3	63	11	17.4	49	30	61.2	75	41	54.6
	T	67	27	40.3	124	31	25.0	114	57	50.0	145	79	54.4
C	♀	19	8	42.1	74	31	41.9	65	31	47.6	68	30	44.1
	♂	15	6	40.0	48	15	31.2	52	16	30.7	71	40	56.3
	T	34	14	41.1	122	46	37.4	117	47	40.1	139	70	50.3
D	♀	26	6	23.0	39	14	35.9						
	♂	34	6	17.6	34	9	26.4						
	T	60	12	20.0	73	23	31.5						

tu = tumor. Iz = Izushi-strain. Ot = Otsu-strain. My = Maruyama-strain.

in Honshu (Izushi and Otsu). On examination, the flies were transferred to different bottles immediately after emergence, and the tumor-bearing flies were calculated every third or fourth day until they died. The results are given in Table 2. The incidence of tumors was about 50 per cent when a tumor female was mated with a wild-type male, while it is about 30 per cent in the reciprocal mating. The difference in tumor incidence occurring between these two crosses is statistically significant. It is on the basis of these data that the occurrence of the maternal effect on the production of tumor is to be suggested.

Remarks

Starting with the work of Stark ('18) who called attention to the significance of the tumorous growth in *Drosophila*, a number of investigators have worked on such tumors occurring in various strains of *Drosophila*. The greater part of the work in the past has made use of *D. melanogaster*. The earlier investigations dealt mainly with the genetic differences, but more recent studies have extended to the effects of environmental factors, such as temperature, crowding and feeding, and X-rays, on the incidence of tumors (Herskowitz & Burdette '51, Plaine & Glass '52, Mittler '52). The literature in this field has been capably reviewed by Scharrer and Lochhead ('50).

Referring to the literature, the tumors occurring in *D. melanogaster* are reported as consistently melanotic, inheritable, and mostly benign. The nature of the tumors found in *D. virilis* resembles that of those in *D. melanogaster* in many respects. But, the tumors of *D. virilis* are characteristic in the point that they appear only in the adult stage, while in *D. melanogaster* they appear in larvae, pupae and adults.

According to Stark ('18, '19, '37), multiple genetic factors in *D. melanogaster* larvae of the strain "lethal 7" give rise to lethal tumors which occur only in the male. In addition to this malignant tumor, a non-lethal hereditary tumor was described by the same author. This benign tumor is not sex-linked. Wilson ('24) reported two *Drosophila* tumors which are due to multiple hereditary factors. In one of them as many as a hundred small tumors were scattered throughout the body. Gardner and Woolf ('49) studied a growth abnormality, "tumorous head," in *D. melanogaster* which seems to be different from the tumors described by Stark. This tumor is said to depend on the action of a recessive (or slightly dominant) sex-linked gene which produces a maternal effect in the egg, and of a semidominant gene located on the third chromosome. Based on the results from some crossing experiments attempted between the tumorous flies and the wild flies of *D. virilis*, it can be stated that there is some evidence to suggest the occurrence of the maternal influence on the incidence of tumor. But, the data presented are rather fragmentary and insufficient for any final conclusion. Further research should be done before the real nature of this tumor can clearly be established.

Summary

Morphological and genetical studies have been made on a tumorous growth found in the wild strain of *Drosophila virilis* which were collected in the suburbs of Sapporo City, Hokkaido. The tumors are benign and melanotic; they take various sizes and shapes. They appear mainly on the mesonotum, scutellum, pleula

and head regions. The tumors appear as pigmented bodies in the imagos within ten to twenty days after emergence. The results of crossing performed between the tumorous strain and the wild strain seem to suggest the occurrence of a maternal influence on the incidence of tumor in the offspring.

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EXPLANATION OF PLATE

Figs. 1-5. Various phenotypic representations of tumors in *D. Virilis*. Numerals put in the right corner of each figure denote the days after emergence of the fly; for instance, 12 d means 12 days after emergence.

Fig. 1. Tumorous growth as a crust-like body developing on mesonotum. Fig. 2. Tumorous growth as a pigmented black speck. Fig. 3. The same as a discrete black body. Fig. 4. The crust-like body changes into the discrete black body which is followed by decrease of pigmentation and finally disappear. Fig. 5. Tumorous growth as melanotic aggregations with fat-like accumulation.

Figs. 6-9. Photographs of the tumors in *D. Virilis*. Figs. 6-7. Tumorous growth as a melanotic aggregation developing on mesonotum. Figs. 8-9. Tumorous growth as a melanotic aggregation with fat-like accumulation.

摘　要

クロショウジョバエにおけるメラニン性腫瘍

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1. 猩々蠅の腫瘍の研究は，現在まで，黄色猩々蠅における種々なる腫瘍系統を用いて行われて来た。著者は 1950 年に，北海道札幌市郊外円山において採集した猩々蠅の集団中より黒猩々蠅のメラニン様腫瘍を見出したので，これについて形態学的及び遺伝学的の研究を始めた。

2. この腫瘍は良性のもので，大きさや形はさまざまである。主として中胸背部，小循板部，側胸部，及び頭部に現われる。また，外部的には羽化後 10 日から 20 日の間に出現する。この点において "tu^h" を除く黄色猩々蠅の各腫瘍系統にみられるものとは異つている。また，腫瘍はメラニン様色素の集成体として現われるもので，この点は "tu^h" とも異つている。なお，この腫瘍を，表現度に従つて一応便宜的に 5 型に区別した (Figs. 1, 2, 3, 4, 5) が，いずれも最後には消失するかあるいは痕跡としてのみ残存する。

3. 4 代にわたる自家交配を行つた結果，腫瘍の発現率が 41 % から 91 % まで，かなり急激に増昇することが判つた。また，腫瘍と出石及び大津の二野生型との間の相互交配の結果から，腫瘍の発現に母性の影響が働いていることを知つた。

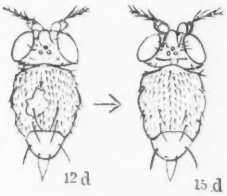

Fig. 1

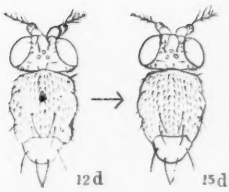

Fig. 2

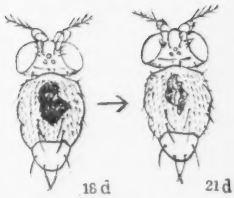

Fig. 3.

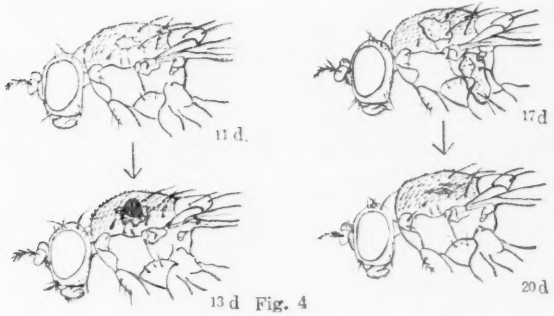

Fig. 4

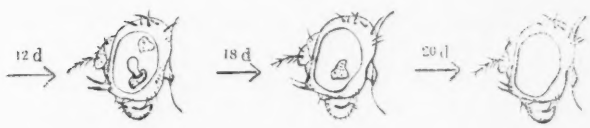

Fig. 5

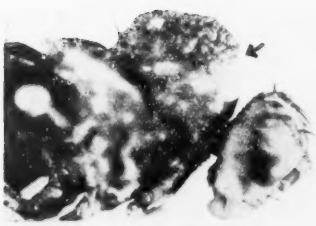

Fig. 6

Fig. 7

Fig. 8

Fig. 9

[GANN, Vol. 45; March, 1954]

EFFECT OF LIVER FEEDING ON THE PRODUCTION OF LIVER CANCER BY 2-ACETYLAMINOFLUORENE.

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Since the discovery of the carcinogenic property of 2-acetylaminofluorene by Wilson and associates[1] in 1941, the dietary influences upon this type of carcinogenesis have been investigated by several workers.[2,3,4,5,6] In connection with the marked inhibiting action of liver feeding on the experimental production of liver cancer by p-dimethylaminoazobenzene[7,8] or o-aminoazotoluol[9], a question may be raised as to whether a similar effect can be produced by liver feeding on the production of liver cancer by 2-acetylaminofluorene. The purpose of the experiments to be reported in this paper was to elucidate this point.

EXPERIMENT

Two groups of normal albino female rats were used in the experiment.

In the first group (liver fed), 20 rats were maintained on the mixture of polished rice (900 g) and dried liver powder (100g). Green vegetables were given occasionally, and water was supplied daily *ad libitum*. The liver powder was prepared simply by drying mashed fresh beef liver, which had been sent through a meat chopper, in an open vessel over a steam bath and by pulverizing, and it was evenly mixed with polished rice just the same as previously described.[6,7,8]

In the second group (control), 30 rats were fed on polished rice without liver powder, also similarly supplemented with vegetables and water.

2-acetylaminofluorene (Eastman Kodak Company) was dissolved in 20 cc of polyethylene glycol (carbowax compound 400, Carbide & Carbonchemicals Corporation, New York) and the solution was evenly mixed with 1 kg of food, and rats were allowed to feed on the mixture *ad libitum*. The amount of 2-acetylaminofluorene was 0.3 g per 1 kg of food (dry weight) for the period of 6 months. After the completion of 2-acetylaminofluorene feeding period, both groups of animals were placed on a normal rice diet without the carcinogen for additional 4 months.

Records were kept of the average amount of food consumed and the average body weight throughout the experiment. Generally speaking the liver fed group appeared to remain in a slightly better physical condition than the control group as is shown in Fig. 1. The average amounts of diet per rat per day were 11.5 g in the liver fed group and 10.5 g in the control respectively.

In expressing the nature of the liver findings, the use of three plus (卅), double plus (卄), plus (+), and minus (−) signs was adopted, indicating respectively, liver cancer, marked nodular hyperplasia or annular cirrhotic changes, slight or moderate nodular hyperplasia and no hyperplasia. These designations closely correspond to those described by Wilson and associates. At the termination, all tumors and grossly abnormal liver tissues were carried through routine procedure for histologic study. Enlargement of the liver of rats in two groups was observed frequently. Hypertrophy and well developed nodular cirrhosis of the liver were not uncommon in most of rats. In addition to increase in fibrous tissue, there was usually extensive increase in small bile ducts. These bile ducts were dilated and formed cysts of various sizes filled with clear yellow or green fluid with the passage of time. And these cysts were seen in the livers of all rats of both groups. Except for these proliferation of cysts, and no enlargement of the spleen, the changes just described are similar to those seen in the livers of rats treated with p-dimethylaminoazobenzene.[7] The complete histological description of these lesions will be reported in another publication.

Approximately, half of the rats of both groups each died early in the course of the experiment without showing noteworthy cancerous changes and these were discarded from the experiment as they add little to the results of the final comparison. The first liver cancer was found in rat in the control group dying 198 days after the beginning of the experiment. And from this time on, 5 other rats of the same group died, most of them showing more or less remarkable hepatic changes. During the corresponding period no rat in the liver fed group died.

Experiment was discontinued at 300 days after the beginning, when all the rats then living were sacrificed for examination. At that time there were 10

Table 1. Liver Changes in the Liver Fed Group.

Rat No.	Duration of Survival Days	Final Body Weight (g)	Liver Weight (g)	Spleen Weight (g)	Liver Findings
1	300	220	64.0	2.5	卅
2	300	205	40.0	1.3	卅
3	300	265	24.3	1.0	卄
4	300	270	21.1	1.2	卄
5	300	310	16.4	0.9	+
6	300	170	14.1	0.6	+
7	300	235	12.9	0.7	−
8	300	255	11.2	1.0	−
9	300	265	10.4	0.9	−
10	300	205	14.4	0.7	−

− : 4 (40.0%), + : 2 (20.0%), 卄 : 2 (20.0%), 卅 : 2 (20.0%)

Table 2. Liver Changes in the Non-Liver Fed Group.

Rat No.	Duration of Survival Days	Final Body Weight (g)	Liver Weight (g)	Spleen Weight (g)	Liver Findings
1	198	195	13.2	1.2	##
2	199	270	20.9	1.4	##*
3	211	200	15.7	0.7	#
4	214	210	33.0	1.0	##
5	219	175	32.5	1.0	#
6	244	200	33.1	1.0	#
7	300	225	40.0	1.0	#
8	300	240	41.7	0.7	#
9	300	135	11.7	1.5	#
10	300	240	21.6	0.6	#
11	300	215	27.2	1.1	#
12	300	205	15.5	0.9	#
13	300	220	44.5	0.9	##
14	300	210	36.5	1.5	##
15	300	190	17.2	0.5	##
16	300	195	33.0	1.9	##

: 9 (56.2%), ## 7 (43.8%).
* With mammary tumor.

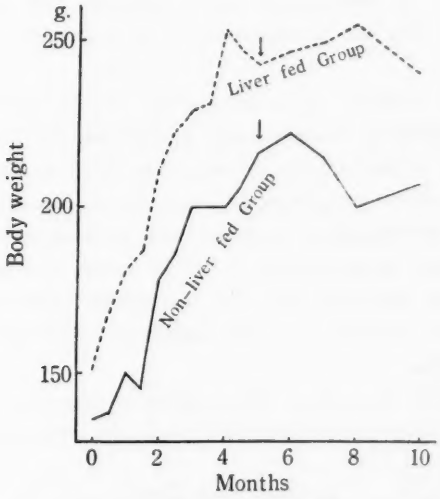

Fig. 1. Curves showing the average body weight of rats.
Arrow shows the time when dye was discontinued.

rats each in the both groups. The results of the above experiment are summerized in Tables 1 and 2, where the body weight, liver and spleen weights and

the nature of liver findings are tabulated for all the rats.

In the liver fed group, 4 (40.0%) of the total 10 rats showed apparently normal liver except cysts, in the 2 cases (20.0%) the liver changes were not advanced beyond the stage of somewhat nodular hyperplasia, and 2 cases the liver showed annular cirrhosis or marked nodular hyperplasia and in the remaining 2 (20.0%) the hepatic changes were advanced to warrent the diagnosis of cancer with nodular hyperplasia.

In contrast to the conditions in the liver fed group, the non-liver fed group (control) included 7 cases (43.8%) of liver cancer accompanying annular cirrhosis and 9 cases (56.2%) of typical annular cirrhosis only.

The above result leaves little doubt as to the inhibiting effect which liver feeding exerts on the production of liver cancer by oral administrations of 2-acetylaminofluorene.

Discussion and Summary

Studies on dietary and genetic influences on the experimental production of liver cancer and other type of tumors in rats by 2-acetylaminofluorene have been made by Wilon and associates, Bielschowsky[2], Morris and associates[10], Paschkis and Stasney[11], Engel and Copeland[5,6], Cantarow, Stasney and Paschkis[12,13] and Harris[3]. Especially, the last-named author indicated that the addition of liver extract (Lilly) to the diet has no appreciable effect upon the production of liver tumors in rats.

In this experiment, however, it is obvious that the liver feeding brought about a considerable inhibition of liver cancer production by 2-acetylaminofluorene, although the degree of the inhibition was not as high as that attained by liver feeding in the liver cancer production by p-dimethylaminoazobenzene. Moreover, the site of tumor development in these rats differed so strikingly from the results of the previous investigators[1,2,5,6,14] in which the induction of tumors in the various tissues are reported. In the present experiment, most tumors were induced in the liver and only one mammary tumor was found in a rat in the non-liver fed group.

At the end of this paper the author takes pleasure in expressing his gratitude to Professor A. R. Moore of the University of Portland, U.S.A., for the generous gift of carcinogen.

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要　旨

2-Acetylaminofluorene 肝癌の生成に及ぼす肝臓飼与の影響

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牛肝飼与が p-Dimethylaminoazobenzene (DAB) による肝癌生成を顕著に抑制する事実は，1938年中原・森らによつて初めて報告されて以来，内外多数の研究者により追証されたところである。一方1941年 Wilson らによつて 2-Acetylaminofluorene (AAF) 飼与による各種の臓器癌の生成が発見された。最近，これら AAF 腫瘍の生成が飼料の組成，ホルモンあるいは動物の系統によつて，著しく左右されることが問題となつてきた。本実験においては AAF 発癌に対する牛肝飼与の影響を検討した。

0.03% の割合に AAF を白米に混じた飼料を与えた雌白鼠30匹 (対照群) と，さらに10% 肝粉末を添加した飼料で20匹の動物 (牛肝添加群) を飼養した。いずれの群でも，AAF の投与は6カ月で打ち切り，さらに4カ月間正常白米食を与えた。

実験の初期に，両群とも各約半数の動物が著変を示さず死亡した。実験開始後198日に至つて，対照群の1匹が肝癌を示し，以後実験終了の300日までに総数16例を剖見した。対照群における肝所見は，7例 (43.7%) が肝癌を，残りの9例 (56.3%) が高度の肝硬変を示していた。これに対し牛肝添加群では，同期間に10例の動物がしらべられた。すなわち2例 (20.0%) は肝癌，2例 (20.0%) は肝硬変を，さらに2例 (20.0%) は軽度の結節状肥大を示し，残りの4例 (40.0%) は肉眼的には正常であつた。

本実験における AAF 腫瘍の発生は，従来の外国研究者の報告と異なり，1例の乳癌 (対照群) を除いては，すべて肝癌であつた。しかし上述の結果は，牛肝飼与が AAF 肝癌生成を，かなり抑制することを示している。このことは肝エキスを用いて抑制効果なしと断定した Harris の報告と，完全に食い違つているが，これらの解決は将来に残されている。

[GANN, Vol. 45; March, 1954]

CATALASE AND URICASE STUDIES ON RATS DURING 2-ACETYLAMINOFLUORENE CARCINOGENESIS.*

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The changes in the activities of catalase and uricase of livers of rats fed p-dimethylaminoazobenzene (DAB) were demonstrated by many investigators.[1~11] In these experiments DAB was administrated with food, in accordance to the original method of Kinoshita.[12]

Wilson and associates[13] has already reported that the liver cancer can be produced with 2-acetylaminofluorene (AAF), and it become of interest to determine the enzymatic activities of rat liver when this carcinogen was administered instead of DAB.

Material and Methods

A group of normal female albino rats, weighing 120–150 g, was maintained on diet containing 2-acetylaminofluorene at a level of 0.03 per cent, and the rats were fed *ad libitum*. The AAF (Eastman Kodak Company) was dissolved in 20 cc of polyethylene glycol (carbowax compound 400): the solution was mixed evenly into the ingredient of diet, consisting of 950 g of polished rice, 50 g of fish powder, 5 g of sodium chloride and 10 cc of cod liver oil.

After 6 months on the experimental diet, animals were placed on the normal rice diet without the carcinogen and were reared for additional 4 months on it. Controls were fed on the same diet without the carcinogen. In both groups, water was freely and green vegetables were occasionally supplied.

Animals were exsanguinated by cardiac puncture under the anesthetization by ether. All livers were removed and inspected. The livers were classified grossly according to the description of Wilson and associates[13] into four grades; no hyperplasia, slight or moderate hyperplasia, marked hyperplasia and liver cancer. In addition, there was usually extensive increase in small bile ducts which followed the formation of cysts of various sizes filled with clear yellow or green fluid. If the liver contained both cancerous areas and relatively normal tissues, the different portions were dissected and handled separately. In most cases, the diagnoses were confirmed by microscopical examination. There was, however, no other tumor except liver cancer in any of rats examined.

* Aided by Scientific Research Encouragement Grant from the Department of Welfare

(a) Determination of Catalase: The method for the determination of catalase activity was as follows: A piece of liver tissues, weighing accurately 0.1 g, was ground up in a mortar and thoroughly extracted with 10 cc of phosphate buffer solution of pH 7, and centrifuged. 0.3 cc of the extract so obtained was diluted with 0.7 cc of the same buffer solution, and its catalase activity was measured gasvolumetrically with the aid of a Battelli-Stern apparatus. The duration of the test was 5 min. at room temperature. The value of oxygen obtained by the action of catalase was recalculated into that at the standard condition.

(b) Determination of Uricase: Enzyme solution was prepared as follows: A piece of liver tissue, weighing exactly 0.5 g, was ground in a mortar and thoroughly extracted with 10 cc of borate solution (pH 8.5), and centrifuged for 5 min. To 0.5 cc of this extract, 2 cc of borate buffer containing 0.4 mg of uric acid and 20 cc of borate buffer was added. After adding 0.5 cc of toluene, each test tube was stoppered tightly and incubated at 37°C for 3 hours. After the incubation, to 5 cc of this solution was added 4 cc of $\frac{1}{12}$ N sulfuric acid 0.5 cc of 10% sodium tungstate solution (Folin-Wu's precipitation method), in succession, after which the solution was filtered. To 5 cc of the filtrate, 4 cc of sodium cyanide solution and 1 cc of arsenophosphotungstic acid was added, the test tube was placed in boiling water bath for 3 min. and it was then cooled under tap water and finally subjected to photocolorimetry.

Results

In Table 1, the results of the catalase activity of the livers are shown, together with their meams and ranges. It may be clearly seen that the liver catalase shows higher slightly than normal at early period of the experiment, when the liver shows hyperplasia at a level of moderate degree. However, in the liver of marked hyperplasia or annular cirrhosis, the catalase was decreased to the half level in its activity. And when the tissue turned cancerous, the catalase activity was reduced definitely. It is reasonable that the catalase activity shows a considerable reduction in the liver of an extensive hyperplasia in which active liver cells may be reduced in number owing to the proliferation of the connective tissues and bilary cysts filled with fluid when compared to the normal. This experimental datum of the liver catalase is in a close agreement with the result obtained in the liver of rats fed DAB.

In Table 2, the results of the uricase activity of the livers are shown, together with their means and ranges. It is seen in the table that no remarkable change was found in the livers which shows on hyperplasia or moderate hyperplasia when compared with that of normal. However, in the liver of marked hyperplasia, a considerable reduction in the activity of the uricase was recognized. When the liver tissue became cancerous, the uricase activity dropped to be

Table 1. Catalase Activity of the Liver of Rats fed AAF.

(Figures given as averages and ranges.)

Liver Findings	No. of Experiments	Liver Catalase O_2 cc.	Catalase Activity*
Normal (Control)	10	5.2 (4.9–5.5)	100
No Hyperplasia	16	5.2 (4.1–6.1)	100
Moderate Hyperplasia	16	5.9 (3.3–7.2)	113
Marked Hyperplasia	18	2.9 (1.1–4.0)	49
Liver Cancer	7	0.8 (0.0–1.6)	14

* Value for normal taken as 100.

Table 2. Uricase Activity of the Liver of Rats fed AAF.

(Figures given as averages and ranges).

Liver Findings	No. of Experiments	Uric Acids Consumed (mg %)	Uricase Activity*
Normal (Control)	20	0.355 (0.327–0.376)	100
No Hyperplasia	5	0.364 (0.341–0.405)	102
Moderate Hyperplasia	14	0.357 (0.318–0.394)	100
Marked Hyperplasia	12	0.307 (0.248–0.346)	86
Liver Cancer	5	0.008 (0.000–0.032)	2

* Value for normal taken as 100.

trace. These data obtained in the experiment by AAF was in close agreement with that of the liver of rats fed DAB.

Summary and Conclusions

An attempt was made in this paper to investigate the activities of catalase and uricase of liver of rats in the course of liver cancer production by AAF feeding. The drastic decrease in the activity of catalase and uricase was recognized in the liver cancer. And the considerable reduction in the activity of both enzymes was seen when the liver showed marked hyperplasia or cirrhosis. However, no remarkable change was occured at the early stage of liver changes in the enzymatic activities. Both catalase and uricase contain iron in the molecules. These experimental data in the activities of enzymes, catalase and uricase, of rats fed AAF have the same tendency to that of rats in the DAB

carcinogenesis.

At the end of this paper it is pleasure to express our appreciation to Professor A. R. Moore of the University of Portland, U.S.A., for his kind gift of 2-acetylaminofluorene.

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要　旨

2-Acetylaminofluorene 肝癌生成過程における カタラーゼ並にユリカーゼについて

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2-Acetylaminofluorene を 0.03% の割合に白米に混じて白鼠に与え，6カ月を経て正常白米食に戻し，さらに4カ月飼育後実験に供した。剖検の結果，肉眼的正常肝から肝癌に至る変化を4段階に分ち，それぞれの病変についてカタラーゼ並にユリカーゼ活性度を測定した。

カタラーゼは最初肝の病変の進行につれて，多少ではあるがその活性度が増したが，肝硬変では正常値に比べて半減し，肝癌では著しく低下した。

ユリカーゼについては，病変の初期には正常肝と変りなく，肝硬変ではかなりの低下を示しさらに肝癌では全くないかあるいは痕跡的存在を示す程度であつた。

これら活性度の変化は p-Dimethylamenoazobenzene 飼与白鼠の肝カタラーゼ並にユリカーゼの消長と殆ど同一の傾向を示している。

[GANN, Vol. 45; March, 1954]

PHOSPHATASE AND ESTERASE OF SERUM AND LIVER OF RATS FED HEPATIC CARCINOGENS.*

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The present study is an attempt to ascertain the relative activity of phosphatase and esterase of serum and liver of rats fed on 2-acetylaminofluorene (AAF) during the course of the production of liver cancer, comparing with that of rats fed on p-dimethylaminoazobenzene (DAB).

MATERIALS AND METHODS

A group of normal adult albino rats was maintained on the AAF rice diet (0.3–0.5 g AAF in 20 cc of polyethylene glycol per 1 Kg of polished rice) for 6 months so as to produce liver cancer. After the period of AAF feeding, the animals were fed on a normal rice diet without the carcinogen for 4 months. Another group of animals was kept on the diet containing DAB (0.6 g per 1 Kg of rice diet) for 4 months by the routine method, and then for an additional month they were fed on the basal diet free from the dye before autopsy. After that, the animals were anesthetized with ether and their blood was collected by cardiac puncture. And then, all livers were carefully examined at the time of autopsy. To show the nature of the liver findings, grossly, four grades of the pathological changes leading to the production of liver cancer were indicated by adopting the four grades of liver findings as described by Wilson and associates[1], that is, i) no hyperplasia, ii) slight or moderate nodular hyperplasia, iii) marked nodular hyperplasia and iv) liver cancer. If the liver contained more than one lesion of the four, the different portions were dissected and handled separately. Representative specimens were taken for histological examination routinely. Except the proliferation of the dilated cysts in the liver of rat fed on AAF, the changes in the liver are similar to those seen in the livers of rats fed on DAB as described in the other report of this issue.[2]

The samples of the liver tissue taken for the examination of the phosphatase and esterase were cut into several pieces.

(a) Estimation of phosphatase: One g of the liver tissue, weighed precisely by the torsion balance, was ground into paste in mortar with emery and water. This paste was suspended in 10 cc of water, and centrifuged. To 1 cc of this

* Aided by a Scientific Research Encouragement Grant from the Department of Welfare.

supernatant suspension, 2 cc of 0.02 M sodium β-glycerophosphate (substrate) and 2 cc of buffer solution (veronal acetate) were added. Then, the determinations of acid, neutral and alkaline phosphatase were made at pH 4, 7 and 9 respectively. To estimate the possible activating effect on the action of the phosphatase, 1 cc of 0.1 M magnesium chloride was also added.

Each mixture, stoppered tightly, was incubated at 37°C for 3 hours. Three kinds of mixture—without substrate, with substrate only and with buffer solution—were used as blank tests. The determination of phosphoric acid liberated from substrate was photometrically carried out by Allen's method.[3)]

(b) Estimation of esterase: A 0.2 g sample of the liver tissue was suspended in 9 cc of distilled water. To this solution, 0.5 cc of tributyrin (0.002 M) and 1 cc of phosphate buffer (pH 7) were added. Then the mixture was incubated at 37°C for 24 hours. The liberated acid was titrated with N/10 NaOH.

Results

(a) Phosphatase

The two carcinogens, AAF and DAB, induced almost a similar change in the content of the phosphatase in relation to the histologic changes.

The mean values and the ranges of the acid and the alkaline phosphatase of

Table 1. Phosphatase Activity of the Serum of Rats fed AAF or DAB.

(Figures given as averages and ranges).

Liver Findings		No. of Experiments	pH 4		pH 7		pH 9	
			A	B*	A	B*	A	B*
	Normal (Control)	9	26 (16–38)	28 (20–52)	33 (17–52)	71 (26–124)	108 (56–172)	176 (60–286)
AAF	No Hyperplasia	11	18 (7–40)	23 (12–38)	27 (8–46)	48 (20– 96)	83 (24–139)	139 (42–262)
	Moderate Hyperplasia	7	16 (8–28)	17 (10–22)	22 (12–32)	31 (14– 56)	44 (20– 50)	87 (32–169)
	Marked Hyperplasia	8	18 (12–24)	21 (18–26)	24 (18–34)	30 (20– 42)	60 (30–106)	97 (36–136)
	Liver Cancer	5	17 (10–20)	19 (11–48)	24 (22–48)	43 (38– 48)	108 (86–139)	192 (150–213)
DAB	No Hyperplasia	9	17 (12–22)	20 (10–34)	28 (12–48)	43 (20– 86)	66 (26–124)	126 (68–231)
	Moderate Hyperplasia	3	13 (10–20)	13 (10–14)	18 (14–22)	25 (20– 32)	49 (26–124)	81 (42–148)
	Marked Hyperplasia	5	19 (12–40)	31 (22–70)	29 (17–72)	63 (38–143)	96 (60–205)	166 (106–327)
	Liver Cancer	7	20 (6–38)	26 (12–48)	33 (16–42)	58 (28–106)	93 (44–193)	161 (80–262)

* Activated Values resulted with the addition of $MgCl_2$

the sera of the animals fed on AAF, on DAB and normal animals are shown in Tab. 1.

As is shown in the table, generally speaking, there was no significant difference in the value of the serum phosphatase between the two groups of carcinogen-fed rats and control rats, when the livers of the former showed marked hyperplasia or cancer. The mean value of the phosphatase and the spread of the range were similar among control, hyperplastic and tumor-bearing animals at pH 4. However, a considerable decrease of the alkaline phosphatase activities was found in the hyperplastic livers.

Table 2. Phosphatase Activity of the Liver of Rats fed AAF or DAB.

(Figures given as averages and ranges).

Liver Findings		No. of Experiments	pH 4		pH 7		pH 9	
			A	B*	A	B*	A	B*
	Normal (Control)	7	293 (240–344)	376 (344–402)	132 (116–180)	208 (180–236)	129 (92–200)	201 (108–250)
AAF	No Hyperplasia	10	272 (264–280)	359 (332–398)	106 (84–124)	177 (144–196)	73 (56– 88)	140 (120–168)
	Moderate Hyperplasia	11	262 (204–307)	336 (262–384)	81 (61– 97)	138 (105–180)	64 (35– 80)	116 (90–150)
	Marked Hyperplasia	9	245 (207–303)	308 (268–384)	82 (60–116)	121 (96–189)	109 (59–222)	159 (98–280)
	Liver Cancer	5	242 (216–267)	291 (276–304)	201 (184–243)	274 (252–288)	730 (588–936)	864 650–1128)
DAB	No Hyperplasia	10	272 (254–276)	372 (340–400)	102 (81–123)	172 (148–207)	62 (32– 92)	130 (108–160)
	Moderate Hyperplasia	4	267 (220–315)	370 (342–428)	98 (80–113)	168 (132–201)	65 (44– 87)	112 (96–144)
	Marked Hyperplasia	11	268 (214–336)	353 (306–394)	112 (82–145)	172 (140–212)	136 (40–287)	190 (96–337)
	Liver Cancer	7	203 (128–244)	301 (220–362)	144 (90–241)	247 (144–440)	420 (332–544)	493 (400–736)

* Activating effect resulted with the addition of $MgCl_2$.

On the other hand, the phosphatase activities of the hepatic tissues of control and of carcinogen-fed rats are shown in Tab. 2 and plotted in Fig. 1. No remarkable change of the value of activities among the control, hyperplastic and tumor tissues was found at pH 4 and 7. But the phosphatase of the liver cancer at pH 9 showed progressively higher mean values and wider ranges than the control and the hyperplastic liver tissues. The mean values at pH 9 were 5.7 times in AAF fed rats, and 3.3 times in DAB fed rats as high as those in the control rats. There was the regular activating effect by magnesium chloride on the action of the phosphatase in all examinations.

These changes obtained in the phosphatase activity in sera and hepatic tissues of

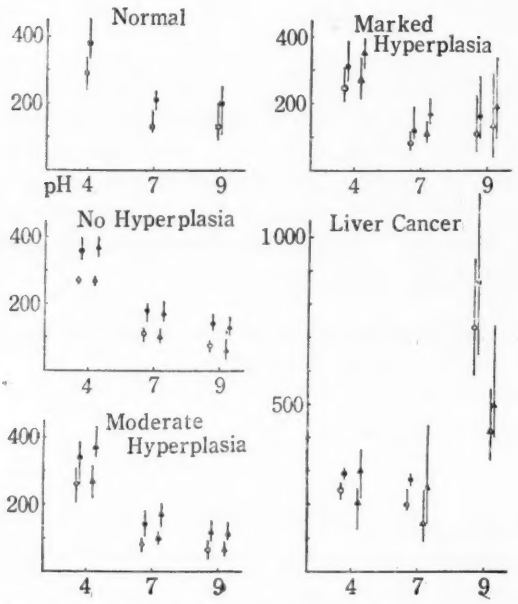

Fig. 1. Graphs showing the phosphatase activities of the livers of rats fed AAF or DAB. (Protted from Tab. 2.)

Circle: AAF, Triangle: DAB, Square: Normal, and activation is represented in solid.

Table 3. Esterase Activity of the Serum of Rats Fed AAF or DAB.

(Figures given as averages and ranges).

	Liver Findings	No. of Experiments	1/10 NaOH Consumed	Esterase Activity*
	Normal (Control)	6	1.8 (1.4–2.2)	100
AAF	No Hyperplasia	9	2.0 (1.7–2.5)	111
	Moderate Hyperplasia	12	2.4 (2.1–3.0)	133
	Marked Hyperplasia	8	2.4 (2.1–2.8)	133
	Liver Cancer	6	2.5 (2.2–2.9)	139
DAB	No Hyperplasia	9	1.9 (1.8–2.1)	106
	Moderate Hyperplasia	4	2.1 (1.8–2.3)	117
	Marked Hyperplasia	6	2.1 (1.8–2.4)	117
	Liver Cancer	7	2.0 (1.7–2.5)	111

* Value for normal taken as 100.

rats are closely similar to those of previous investigators.[7,9]

(b) Esterase

The mean values and the ranges of the esterase activities of sera and hepatic tissues are shown in Tabs. 3 and 4, respectively. In Tab. 3, a considerable shift in the serum esterase activities is shown in the carcinogen-fed rats when compared with the control rats. Especially it is more remarkable in the rats fed AAF than in the rats fed DAB. And no remarkable change is recognized in the serum esterase activities between hyperplastic rats and tumor rats. In Tab. 4, however, a drastic decrease, even to one half of the control, is found in the esterase activities of the liver when the tissues have become cancerous. And no remarkable change is recognized between the hyperplastic tissues and the controls.

Table 4. Esterase Activity of the liver of Rats fed AAF or DAB.
(Figures given as averages and ranges).

Liver Findings		No. of Experiments	1/10 NaOH Consumed	Esterase Activity*
Normal (Control)		8	5.8 (5.3–7.0)	100
AAF	No Hyperplasia	14	5.8 (5.1–7.0)	100
	Moderate Hyperplasia	15	6.9 (5.9–8.8)	119
	Marked Hyperplasia	13	6.1 (5.2–7.0)	105
	Liver Cancer	5	3.0 (2.0–4.2)	52
DAB	No Hyperplasia	7	6.2 (5.7–7.3)	107
	Moderate Hyperplasia	4	5.7 (4.9–6.2)	98
	Marked Hyperplasia	12	5.8 (4.9–7.1)	100
	Liver Cancer	8	3.1 (2.3–4.7)	54

* Value for normal taken as 100.

Discussion and Conclusions

Since the discovery of the carcinogenesis of the azo-compounds by Sasaki and Yoshida[4], and Kinoshita[5], the phosphatase and the esterase activities of the sera and hepatic tissues, non-cancerous or cancerous, of the rats fed these chemicals have been investigated by many researchers.[6,7,8,9,10,11] In this report, the relative activities of the phosphatase and the esterase of the serum and the liver of rats fed on AAF are demonstrated comparing with that of rats fed on DAB.

Generally speaking, the results obtained in the rats fed on AAF were closely similar to those of previous investigators who studied the rats fed on DAB. No difference of the phosphatase content of the serum was indicated between the tumor bearing rats and control rats in any examination at pH 4, 7, and 9. There was no appreciable difference in the acid phosphatase content (pH 4) of the liver tissues among the control, the hyperplastic and the tumor bearing rats. But in the hepatic tumors, a striking rise in the alkaline phosphatase (pH 9) was demonstrated.

There was a slight increase in the esterase content of the serum in the process of the development of the liver cancer. The mean value of the esterase activity of the hepatic tumors was one-half of that of the normal liver.

At the end of this paper we take pleasure in expressing our gratitude to Professor A. R. Moore of the University of Portland, U. S. A., for his kind gift of 2-acetylaminofluorene.

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要　旨

肝癌生成過程における白鼠の血清並に肝の Phosphatase および Esterase について

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2-Acetylaminofluorene (AAF) を動物に与えて肝癌を生成する過程における白鼠血清および肝の Phosphatase と Esterase の活性度をしらべた。この際，p-Dimethylaminoazobenzene (DAB) による肝癌を前者の場合と比較した。

実験結果は表示の如くであるが，総括的にいえば，AAF あるいは DAB 投与による肝癌生成過程におけるそれぞれの白鼠の血清と肝の Phosphatase, Esterase 作用は，肝病変に応じ殆ど同じようであつた。

すなわち肝癌生成過程における白鼠の血清 Phosphatase は試験された範囲の pH 域 (pH 4, 7 ならびに 9) では対照動物に比べて顕著な差がみとめられなかつた。肝組織 Phosphatase は pH 4 の場合には対照に比べて大差なく，しかし pH 9 の場合には肝癌 Phosphatase が著しく増量した。このことは DAB 投与白鼠についてなされた従来の諸報告とほぼ一致している。

血清 Esterase 作用は AAF あるいは DAB を与えた動物では対照に比してやや強く，殊に AAF 肝癌動物ではかなり高位を示した。しかし肝組織 Esterase 作用は病変の進行に関係なく不変で，さらに肝癌では半減することがみとめられた。

[GANN, Vol. 45; March, 1954]

ASPARAGINASE ACTIVITY IN TISSUES OF THE RATS FED WITH CARCINOGENS, p-DIMETHYLAMINOAZOBENZENE AND 2-ACETYLAMINOFLUORENE.

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(Director: Prof. Sanji Kishi)

In previous papers the liver asparaginase activity of the rats fed on p-dimethylaminoazobenzene (DAB) has been reported by Kishi and the present author. In that work the activity has been studied only on the liver, which is the most susceptible organ to that carcinogen. As we showed, a few weeks after the DAB feeding the remarkable lowering of the asparaginase activity of the liver was found, in comparing to that of control rats. This phenomenon was seen as long as DAB was incorporated to the diet. In the later period when the liver lesion has developed, the activity remained still very low. When, after the prolonged DAB feeding, the rats were removed from the DAB diet and were put on the normal diet for a while, the liver asparaginase recovered nearly to the normal activity, notwithstanding the pathological changes in livers. Once the liver lesion has progressed to neoplastic stage, it showed always very low activity regardless of whether the host animals were left on DAB diet or discontinued form it.

In continuation of the above mentioned work the author studied the asparaginase activity of several organs other than the liver, which are less susceptible to DAB, during the feeding with DAB diet and after its interruption.

In the present study another carcinogen, 2-acetylaminofluorene (AAF), was introduced for comparison and special attention was paied for the multiple carcinogenic effects of AAF, which may produce some different influence on the asparaginase activity of the tissues.

METHODS

Experimental animals.—DAB rats: Albino rats weighing about 120 g were placed on a DAB diet, consisting of 1 kg rice and 0.6 g DAB (Kokusan Chemical Works) in 20 g soy bean oil for 120–150 days. One part of the above DAB rats were returned to normal diet (rice grains) successively for 30–40 days after the long DAB feeding above mentioned.

AAF rats: The rats were fed on AAF diet consisting of 1 kg rice and 0.3 g AAF (Eastman Kodak Company) in 20 g polyethylenglycol for 150–180 days. A

part of AAF rats were later removed from AAF diet and were placed on normal diet for 90–130 days.

For the early experimental stage of AAF feeding, the rats were investigated weekly. A part of rats were given the AAF diet consisting of rice, AAF and soy bean oil instead of polyethylenglycol. For the comparative study, a group of rats were fed on rice containing solely polyethyleneglycol or soy bean oil free from AAF.

Normal rats: Untreated rats of similar age were placed on normal diet (rice grains).

All experimental animals were allowed to take food and water ad libitum with occasional supply of dried fish and green vegetables.

Enzyme preparation.—The hepatic tissues, kidney, brain and testis were excised from the animal immediately after decapitation and blotted dry, weighed and homogenized in the presence of some water. To the tissue paste water was added up exactly to 10 or 20 times of its original tissue volume (cc) calculated from the weight (g) and was thoroughly shaken. The homogenate thus obtained was used as enzyme preparation without any further procedure such as filtration or centrifugation.

Estimation of asparaginase activity.—Digestion mixtures were prepared as follows. In a large thick wall pyrex test-tubes 2 or 4 cc of enzyme preparation (2 cc of hepatic tissues in 20 times dilution, 2 cc of kidney in 10 times dilution, 4 cc of brain in 10 times dilution, 4 cc of testis in 10 times dilution), 3 cc of 0.1 M phosphate buffer at pH 8, 2 cc of 0.1 M asparagine solution as substrate and last of all 3 or 1 cc water was added to make the whole volume of mixtures up to 10 cc, and incubated for 24 hours at 38°C under toluene layer, stoppered tightly.

The determinations of freed ammonia which has been evolved from asparagine were made by the method described in detail elsewhere. From these data were subtracted those of blank tests and ammonia nitrogen was calculated in mg, which was liberated by enzyme preparation equivalent to one gramme fresh tissue.

Results

Results in DAB rats.—The rats fed with DAB diet more than 120–150 days continuously until the determinations were carried out, the activity of asparaginase of hepatic tissues were extremely low in comparison with the livers of normal rats of about the same age. At this stage the pathological changes were found more or less in the hepatic tissues. When the DAB diet was removed after long period of feeding the rats were placed on normal diet for several weeks, the asparaginase activity of abnormal but non-cancerous liver recovered, approaching to normal value. The activity of hepatoma, on the

contrary, remained always very low, in spite of the feeding conditions have been altered (Table 1, Chart 1).

Table 1. Asparaginase activity at pH 8 in several organs of rats fed with carcinogens, DAB and AAF.

Numbers, mean value with maximum and minimum values, represent ammonia nitrogen in mg, which was evolved from asparagine by enzyme preparation equivalent to 1 g of fresh tissue at 38°C for 24 hours, and were used in Table 2 and Charts 1-6.

Numbers in parenthesis represent the experimental numbers for the determination of the corresponding data.

		Hepatic tissues		Kidney	Brain	Testis
Normal rats		11.9 25.6–3.9 (33)		9.4 11.0–7.3 (28)	1.3 1.5–1.1 (26)	3.6 4.0–3.2 (10)
DAB rats	Long period of feeding	Pathological but non-cancerous	Hepatoma	6.7 8.8–5.5 (7)	1.1 1.3–0.9 (6)	3.5 3.7–3.3 (4)
		1.3 1.8–0.7 (7)	—			
	Interrupted after long period of feeding	7.2 16.0–3.3 (9)	1.6 2.4–0.8 (6)	8.2 10.0–6.7 (9)	1.3 1.5–1.2 (9)	3.1 3.9–1.5 (10)
AAF rats	Long period of feeding	1.0 1.8–0.6 (6)	—	8.4 9.9–7.2 (3)	1.2 1.2–1.1 (6)	—
	Interrupted after long period of feeding	7.5 16.2–2.7 (18)	1.0 1.1–0.8 (3)	6.8 9.1–5.5 (16)	1.2 1.4–1.1 (14)	—

Table 2. Asparaginase activity at pH 8 of the AAF rats.

The rats fed with AAF diet for 150–180 days and then were interrupted for 90–130 days. The data are illustrated in Chart 3.

Pathological but non-cancerous liver	No. of exp.	Ammonia-N mg. Mean value with max. and min. values
No hyperplasia	5	8.5 11.3–5.3
Nodular hyperplasia	7	8.2 16.2–3.8
Marked nodular hyperplasia	8	5.7 9.9–2.7

Chart 1 Hepatic tissues

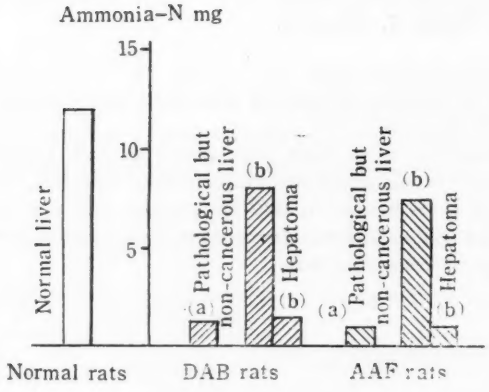


Chart 4 Kidney

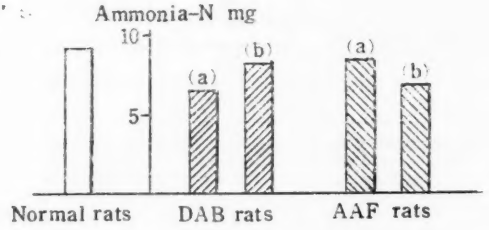


Chart 5 Brain

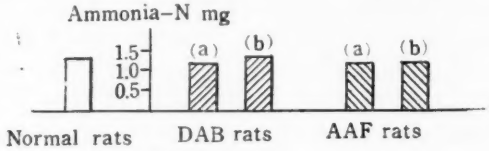
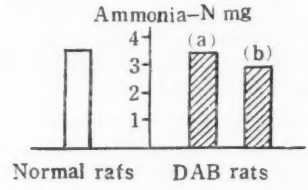


Chart 6 Testis



Asparaginase activity at pH 8 of several tissues.

Shaded area (a) represents the activity of tissues of rats fed with the carcinogenic diet for a long period of time continuously until the experiment was carried out. Shaded area (b) represents that of rats after prolonged feeding of the carcinogenic diet once interrupted and were placed on normal diet for several weeks.

The asparaginase activities of kidney, brain and testis of rats fed on DAB for more than 120 days, until the determination was carried out, or of DAB rats in which the azo dye diet was discontinued and returned to normal diet for several weeks, were not markedly different from normal tissues (Table 1, Charts 4, 5, and 6).—As the scale of ordinate in Charts 5 and 6 has been represented reasonably enlarged, slight difference of activities shown was considered not noteworthy.

Results in AAF rats.—The asparaginase activity of the livers of the rats fed with AAF diet was markedly lowered within 2–3 weeks of experimental days and reached at the end of fourth week to the lowest, and that of the rats fed with diet containing solely polyethylenglycol or soy bean oil, which has been used as the solvent of AAF in case of AAF experiment, showed no difference between control rats (Chart 2).

When the rats were placed on the AAF diet more than 150 days continuously, until the determinations were carried out, the activity of hepatic tissues was

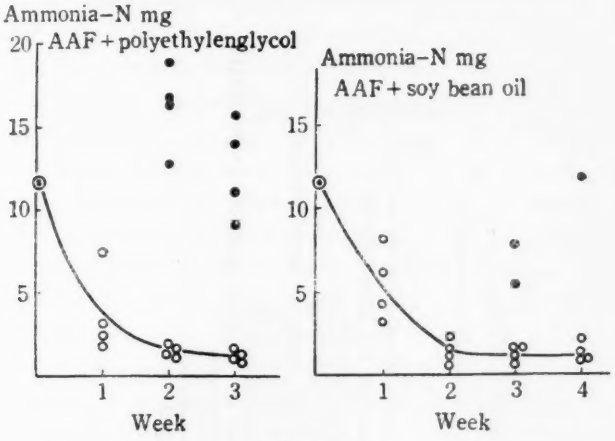


Chart 2

Activity of liver asparaginase at pH 8 of rats in the early period of AAF feeding. Where AAF diet was prepared by adding AAF to the normal diet after the solution has been made by dissolving AAF in polyethylenglycol or soy bean oil respectively.

Double circles: the average value of normal rats, in short the starting value.

Open circles: each observed value on corresponding feeding weeks.

The curves were traced the weekly mean value and represent the lowering of activity every week from the time of AAF feeding has been started.

Solid circles: each observed value of the rats fed with solely polyethylenglycol and soy bean oil which added to basal diet respectively.

markedly low reaching to the minimum value, notwithstanding the hepatic lesions were observed more or less at this time (Table 1, Chart 1).

The rats, in which the AAF diet was discontinued after a long period of time and were fed with normal diet successively more than 90 days, the activity of pathological but non-cancerous liver recovered about to 2/3 of the normal value. In this case the activity of hepatoma nodulus remained still very low (Table 1, Chart 1). The pathological but non-cancerous livers of AAF rats just mentioned were classified further in detail histologically, according to the idea of Wilson et al., in liver of no hyperplasia, nodular hyperplasia and marked nodular hyperplasia. In the first two groups of the livers, the activity was more than 2/3 that of normal livers and in the last group about 1/2 of control value (Table 2, Chart 3). Some specimens of the last group had multiple cysts histologically.

The asparaginase activity of kidney and brain of rats, fed on AAF diet for long period of time, showed no fundamental difference between the respective tissue of normal rats, regardless of whether the rats were fed continuously with AAF diet or discontinued and put on normal diet (Table 1, Charts 4 and 5).

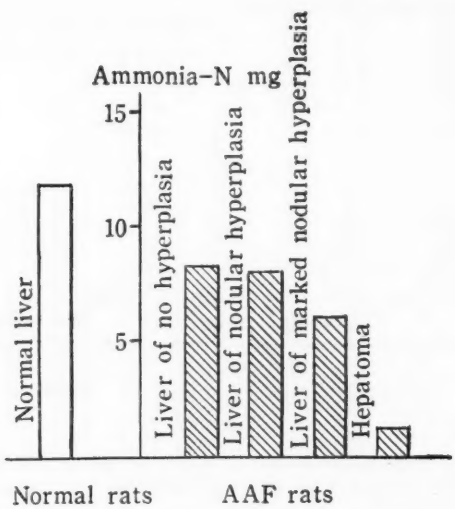


Chart 3

Asparaginase activity at pH 8 of hepatic tissues of the AAF rats.
The rats fed with AAF diet for 150–180 days and then interrupted for 90–130 days. In this Chart the pathological but non-cancerous liver were classified further in detail than they were represented in Table 1 and Chart 1.

Discussion

When the rats were put on the AAF diet, the marked depression of the liver asparaginase activity was observed at 3–4 weeks from the begining of the experiment. The quite similar result has been found by Kishi and the present author previously, in the rats at the early period of DAB feeding.

Miller et al. confermed that, when the rats fed with azo dye diet, protein bound dye was formed in the liver and in case of DAB rats the amount of azo protein reached to the maximum at the end of the fourth week of the experiment; thus they posturated modification or deletion of protein (enzymes) took place. So the deletion of the liver asparaginase also might have been caused by combining with azo dye, thus lowering the activity. They found also AAF bound protein in the livers of rats fed on AAF by using C^{14} labeled compound. According to my determination of the activity of liver asparaginase of AAF rats, it might be supposed that the maximum amount of Miller's protein-bound AAF in livers might have been reached within 4 weeks of feeding, because the activity of liver asparaginase diminished to the minimum value when the rats have been fed with AAF diet for about 4 weeks. After prolonged feeding with DAB or AAF diet, which have been maintained until the determination was carried out, the asparaginase activity of the livers was extremely low, and when the rats have been removed from the carcinogenic diet, the asparaginase recovered its activity, although not compretely, in spite of some pathological changes in the livers, which have developed more or less during this time. Once the livers turned to cancer, the cancerous tissue showed always very low activity, not related to whether the hosts were remained on the carcinogenic diet or removed from it. Cancer tissue seems to behave indifferently to the carcinogen, so to speak, even if it was induced by the same carcinogen.

After the interruption of the AAF diet, AAF might be released by and by from protein-bound AAF in the hepatic tissues just as Miller et al. previously confirmed on their DAB fed rats.

And the tissues other than the livers of the rats fed carcinogen diet for a long period of time showed no marked differences from the respective tissues of control rats.

The liver is the major site of tumor formation, when the rats were placed on DAB diet. AAF is different from DAB in respect of its multiple carcinogenic effect, but still the liver is one of the major sites of tumor formation. Therefore, in the rats under these conditions the enzyme systems of the liver might be expected to be affected in the first place. One of the enzyme inhibiting actions of the carcinogen has been expressed in the asparaginase as described above.

Summary

The asparaginase activities of several tissues of rats fed with carcinogens, p-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorene (AAF), were studied.

The activity of liver asparaginase was very low after the long period of carcinogen feeding. When the carcinogenic diet was discontinued after a prolonged feeding, the activity of liver asparaginase recovered, tending to the value of normal rat liver, notwithstanding the liver lesions, except hepatoma.

The activity of hepatoma tissue was always extremely low whether it has been induced by DAB or AAF and under any feeding condition.

In the short period of AAF feeding the liver activity was diminished weekly and reached to the lowest within 3–4 weeks. The result is quite similar to what has been publicated elsewhere about the DAB fed rats.

The asparaginase activity of tissues other than the liver, such as kidney, brain, testis of DAB fed rats were studied. No remarkable difference was found compared to normal corresponding tissues. The activity of kidney and brain asparaginase of AAF fed rats also showed similar results.

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要　旨

発癌物質経口投与ネズミの臓器アスパラギナーゼについて

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バターエロー (DAB) およびアセチールアミノフルオレン (AAF) をそれぞれ経口投与した鼠の肝およびその他の臓器のアスパラギナーゼ (asp) の活性度を検討した。DAB 投与鼠の肝 asp の活性度に関する既報の事実を再確認し，さらに AAF 投与鼠についても全く同様な結果を得た。すなわち AAF 投与の初期実験において鼠肝の asp 活性度は正常動物にくらべて顕著に下り，約4週で最低値に達する。

AAF を長期投与し測定の直前までつづけた鼠の肝では asp 活性度は極わめて低い。この時期の肝は病変肝である。長期投与の後，AAF 食を中絶し正常食にもどして飼育した鼠の肝の asp 活性度は正常値に近づいている。すでに肝癌になつた部の活性度には回復現象はない。

Miller らの提唱するアゾ色素投与により肝蛋白結合アゾ色素が生じ肝の蛋白 (酵素) の変性を来すという説に思いあたる。AAF 投与の場合も全く同様に AAF が肝酵素に結合し変性をおこすものと想像する。この現われの一つとして著者のみた如く肝 asp の活性度が AAF 投与で低下したものと思う。

肝以外の臓器，脳，腎等で asp の活性度を測定したが正常との差異は論ずることができなかつた。すはわち多発性の発癌物質といわれる AAF と癌を特異的に肝に好発させる DAB を用いた場合との差は結局認められなかつた。

(文部省科学研究費による)

擱筆に当り実験動物に関し多大の御援助を賜わつた本学教授森和雄先生に，また発癌物質について大変に御世話下さつた東京医科歯科大学教授太田邦夫先生に深甚なる謝意を表する。

[GANN, Vol. 45; March, 1954]

ENZYMATIC DESAMINATION OF NUCLEIC ACIDS IN LIVER OF RATS FED ON p-DIMETHYLAMINOAZOBENZENE.

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In connection with the series of our enzymological studies of esterase, cathepsin and asparaginase of the liver of the rats fed on hepatic carcinogen, we became interested in some other enzymes.

For the determinations of desaminase activities ribosenucleic acid and desoxyribosenucleic acid were employed this time, though these latter have, in their molecular configurations, many sites susceptible to enzymatic action.

About these enzymes extensive and noteworthy articles were already publicated by Greenstein et al.

METHODS

Experimental animals.—1) DAB rats. Albino rats of mixed strains, weighing about 100 g, were fed on a diet consisting of 1 kg rice and 0.6 g p-dimethylaminoazobenzene (DAB) in 20 g soy bean oil for about 150 days, then the feeding was interrupted and replaced on normal diet for several weeks.

2) Liver powder fed rats. Untreated rats fed with diet consisting of 900 g rice, 100 g beef liver powder and 20 g soy bean oil for several weeks beforehand.

3) Normal rats. Untreated rats were kept on a normal diet (rice grains) for several weeks before the experimental use. The rats of the latter two groups were employed for control, selecting the similar age to that of DAB rats.

All the rats were allowed on food and water ad libitum and supplemented by green vegetable occasionally. Dried fish meal was given weekly to rats of Groups 1 and 3, but not of Group 2.

Enzyme extract.—The hepatic tissues were excised from the animal immediately after decapitation and exsanguination, weighed and homogenized. Then the distilled water was added to the tissue paste exactly to 5 or 10 times its original tissue volume (cc) calculated from the weight (g) and was thoroughly shaken. This waterly suspension was employed as enzyme preparation.

Estimation of desaminase activity for ribosenucleic acid.—Nucleic acid from yeast (E. Merck) was employed as substrate, namely, ribosenucleic acid (RNA). 3 cc of 2 per cent solution of RNA sodium salt was transfered into a large pyrex test tubes and mixed with 3 cc of 0.1 M buffer solution—citrate buffer (pH range 3 to 4), phosphate buffer (pH range 5 to 8), glycocoll-NaOH buffer (pH

range 9 to 12)—and 2 cc of enzyme preparation in 5 times dilution (equivalent to 0.4 g of original fresh tissue) and 2 cc of water to make the whole volume up to 10 cc. The digestion mixtures, overlayed with some toluene and stoppered tightly, were stored at 38°C for 24 hours. The ammonia produced during the digestion was determined by Folin's method. The data were corrected for the blank test of buffer solution mixture without substrate.

Estimation of desaminase activity for desoxyribosenucleic acid.—Desoxyribosenucleic acid (DNA) employed as substrate in this experiment has been prepared practically pure from calf spleen in our laboratory according to Feulgen's method.

As substrate, 3 cc of 2 per cent solution of DNA sodium salt was generally used; its amount was increased, however, if the enzyme in tissue extract was too active and substrate was not enough to satisfy the maximal activity.

Other than the above mentioned substrate, the digestion mixtures were composed of 3 cc of 0.1 M buffer solution—citrate buffer (pH range 5 to 7), glycocoll-NaOH buffer (pH range 8 to 10)—and 2 cc of enzyme preparation in 10 times dilution (equivalent to 0.2 g of original fresh tissue) and 2 cc of water, which were mixed in a large pyrex test tubes. The further procedure was the same as described above.

The activity was presented as follows: ammonia nitrogen in mg, which has been evolved from RNA or DNA by the enzyme action of extract, equivalent to 1 g of fresh hepatic tissue, stored at 38°C for 24 hours.

Results

Activity of the desaminase for nucleic acids in rat liver was studied in the course of liver cancer production of DAB fed rats.

Two maximum levels at pH range 5 to 6 and 10 to 11 were observed in pH-activity curves of RNA-desaminase. Normal liver and that of rats fed on beef liver powder showed the quite similar forms (Table 1, Charts 1 and 2). So far as the present enzyme was concerned, the special diet, beef liver powder, has shown no effect on its activity.

The peak in the acid side of pH-curves once increased according to the intensity of pathological changes of the liver of DAB fed rats, namely, in macroscopically normal liver, liver with uneven surface; then began to decrease in cirrhotic liver and hepatoma, but the latter kept still some activity compared to that of the normal liver.

The peak in the alkaline side elevated its height gradually, according to the grade of liver lesions and markedly in hepatoma (Table 1; Charts 3, 4, 5, 6, and 7).

Table 1

			pH	3	4	5	6	7	8	9	10	11	12
Control rats			Normal rats	0.34 0.53–0.18 (3)	0.55 0.88–0.35 (5)	1.46 1.72–1.09 (6)	1.47 1.68–1.05 (8)	0.94 1.23–0.6 (8)	0.57 0.81–0.25 (7)	0.58 1.02–0.32 (8)	0.90 1.19–0.59 (8)	1.02 1.51–0.74 (8)	0.26 0.88–0 (8)
			Liver powder fed rats	0.38 0.48–0.32 (5)	0.53 0.6 –0.49 (5)	1.36 1.65–0.95 (5)	1.35 1.68–0.98 (5)	0.74 1.05–0.28 (5)	0.48 0.63–0.35 (5)	0.37 0.46–0.28 (5)	0.91 1.19–0.6 (5)	0.97 1.23–0.7 (5)	0 (5)
DAB rats	Liver findings		Macroscopically normal	0.46 0.56–0.35 (4)	0.70 0.77–0.67 (4)	1.80 1.89–1.65 (4)	1.73 1.79–1.65 (4)	1.28 1.37–1.16 (5)	0.74 0.84–0.56 (4)	0.53 0.95–0.39 (4)	1.0 1.61–0.63 (7)	1.14 1.65–0.91 (5)	0 (4)
			Uneven surface	0 (1)	0.46 (1)	1.84 2.03–1.61 (3)	1.88 2.1 –1.68 (4)	1.30 1.54–1.19 (7)	0.81 0.84–0.77 (4)	1.05 1.3 –0.74 (4)	1.05 1.23–0.81 (8)	1.30 1.54–1.05 (4)	0.84 1.37–0.49 (4)
			Cirrhotic	0.31 0.53–0 (5)	0.60 0.7 –0.49 (5)	1.67 1.89–1.42 (8)	1.6 1.72–1.47 (7)	1.1 1.33–0.81 (10)	0.7 0.88–0.49 (8)	0.78 1.16–0.35 (8)	1.32 1.75–0.88 (12)	1.5 1.86–0.95 (7)	0.43 1.19–0 (8)
			Hepatoma	0.11 (1)	0.63 (1)	1.47 (1)	1.41 1.75–0.81 (4)	1.0 1.19–0.7 (6)	0.79 0.88–0.67 (4)	1.15 1.75–0.88 (5)	2.15 2.38–1.89 (6)	2.23 2.45–1.89 (4)	0.56 1.33–0 (4)

Activities of liver desaminase for ribosenucleic acid of rats on DAB.

Numbers, mean value with maximum and minimum values, represent ammonia nitrogen in mg, which was evolved from the substrate by enzyme preparation equivalent to one g of fresh tissue at 38°C for 24 hours, and were used in Table 2 and Charts 1–11.

Numbers in parenthesis represent the experimental numbers for the determination of the corresponding data.

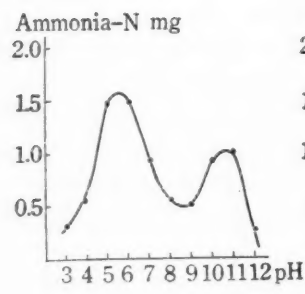

Chart 1
Normal liver

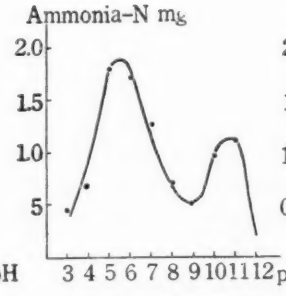

Chart 3
Macroscopically normal liver

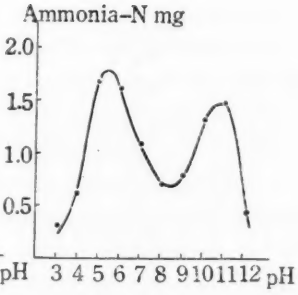

Chart 5
Cirrhotic liver

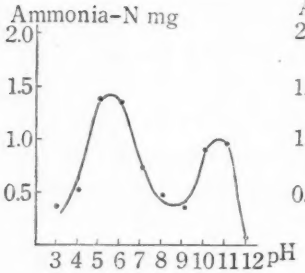

Chart 2
Liver of the liver powder fed rats

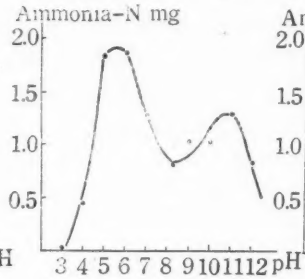

Chart 4
Liver of uneven surface

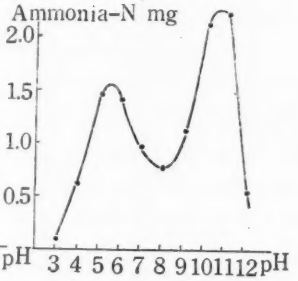

Chart 6
Hepatoma

Control rats

DAB rats

Table 2

	pH	5	6	7	8	9	10
Normal rats		0.14 0.35–0 (8)	2.43* 3.01–1.75 (9)	0.57 0.91–0.35 (8)	0.29 0.49–0 (8)	0.92 1.96–0.14 (8)	0.04 0.14–0 (8)
DAB rats	Cirrhotic liver	1.02 1.47–0.56 (10)	3.92* 4.9 –3.36 (10)	1.55 2.1 –0.7 (10)	1.0 2.8 –0.21 (10)	2.12 2.94–0.7 (12)	0.26 0.56–0 (10)
DAB rats	Hepatoma	0.68 1.47–0.21 (4)	3.22* 5.18–1.47 (4)	1.37 2.03–0.56 (4)	0.95 1.82–0.42 (4)	2.38 4.2 –1.61 (5)	0.37 1.05–0 (4)

Activities of liver desaminase for desoxyribosenucleic acid of rats on DAB.

Numbers represent in the same way as described under Table 1.

The digestion mixtures of the experiment at pH 6 contained high concentration of substrate, namely, 5 cc of 2 per cent DNA sodium salt solution instead of usual 3 cc, and 3 cc of buffer solution and 2 cc of enzyme preparation in 10 times dilution. So the mixtures were 10 cc as a whole without adding any water.

* The data represented in Chart 11, not in Charts 8, 9, and 10.

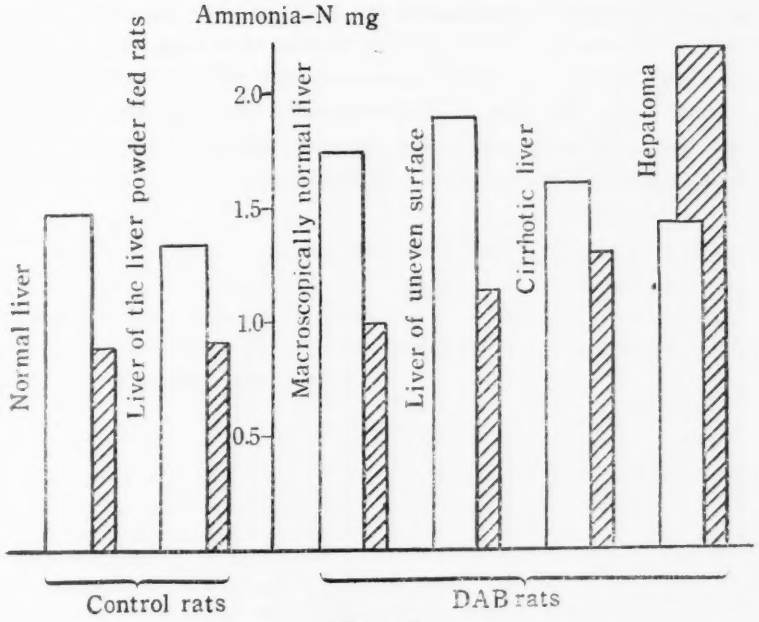

Chart 7

Activities of liver desaminase for ribosenucleic acid of rats on DAB.

Unshaded areas represent the activities at pH 5, and they increased in pathological but non-cancerous liver and decreased in hepatoma.

Shaded areas represent the activities at pH 11, they increased stepwise according to the grade of liver lesions, especially in hepatoma.

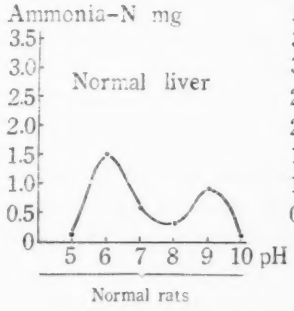

Chart 8

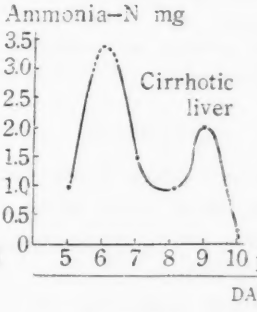

Chart 9

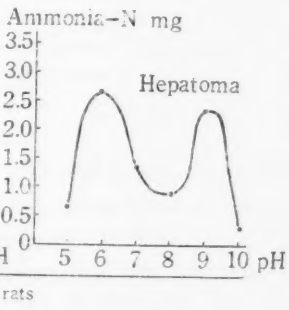

Chart 10

pH-Activity curves of liver desaminase for desoxyribosenucleic acid of rats on DAB.

The peak at pH 6 of the curve in Chart 9 was illustrated with dotted line, where the condition of the digestion mixture was different in respect to its high concentration of substrate as described under Table 2.

The peak at pH 6 in Charts 8 and 10 was plotted against the data, which were obtained according to the method by using usual digestion mixtures as described in text, not under Table 2.

The pH-activity curves of desaminase for DNA showed also two peakes at pH 6 and 9 in its curves. Cirrhotic liver showed very high level at pH 6 and decreased when the liver has turned cancerous, but remained still higher than that of normal liver. The peak at pH 9 represented very high value in abnormal livers, but the difference of activities between cirrhotic liver and hepatoma were not detectable (Table 2; Charts 8, 9, 10 and 11).

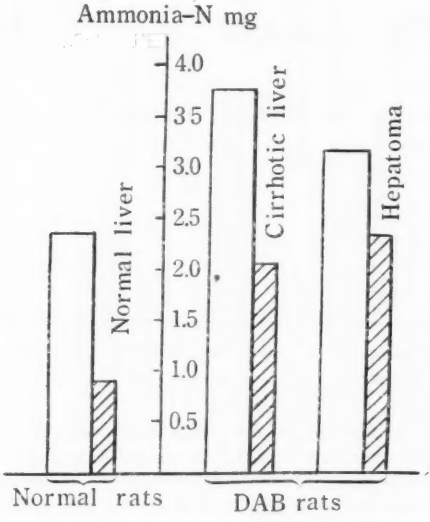

Chart 11

Activities of liver desaminase for desoxyribosenucleic acid of rats fed with DAB.

Unshaded areas represent the activities at pH 6.

Shaded areas represent the activities at pH 9.

Discussion

We have chosen this time in our series of enzymological studies on carcinogenesis as substrate a highly complicated compound, such as nucleic acids, which may have many susceptible sites in the molecular configuration, susceptible to numerous enzymatic cleavage actions.

In the molecule of the nucleic acids, therefore, there might have occurred many different enzymatic actions at the same time or successively in coexistence of the hepatic tissue extract. On this occasion we paid attention merely to nucleic acid desaminases activity, so there might have been left many other problems behind, which require further study. Anyway we introduced nucleic acid, which was considered to have close relation to the cell proliferation, as substrate and investigated its desaminase activity, and confirmed that the activity

increased more markedly than that of normal livers when the liver has suffered from some lesions.

Summary

Activities of desaminases for ribosenucleic acid and desoxyribosenucleic acid of the hepatic tissues of rats during p-dimethylaminoazobenzene carcinogenesis were investigated.

pH-Activity curves of these enzymes had two pH optima on acid and alkaline sides, where the activity level could be easily distinguished from each other.

These enzymes contained in pathological livers had always higher activity than those of control livers and were markedly notable in the case of hepatoma, especially at alkaline side optimum.

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要　旨

アゾ色素投与白鼠肝の核酸脱アミノ酵素について

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バターイエロー投与によつて白鼠に肝癌が実験的に生成される過程において，肝酵素の活性度を調べることは肝の正常機能が癌化するに伴つておこる変化を酵素学的に追究することである。

白鼠にバターイエローを投与し，約150日経過し後正常食に数週間もどしたものの肝を選んだ。これを肉眼的に観察して肉眼的正常，表面不平滑，硬変肝，肝癌に区別して取り扱つた。対照として米食の正常白鼠の肝および牛肝未飼与白鼠の肝を用いた。酵素液は水均質液を用い，緩衝液と基質としてRNA (メルク製) あるいはDNA (牛脾より分離) の2%溶液を用いた。これら混合液を38°C, 24時間保存後，Folin氏法により遊離したアンモニアを定量して脱アミノ作用の活性度とし，生組織1g当りに算出して比較した。この際白紙試験によつて得た値を差引いた。

活性度をpH曲線によつてみるとRNA脱アミノ酵素では二つの極大値 (pH 5-6 および 10-11) を認めた。酸性側の極大値においては正常肝よりDAB投与白鼠の肝は活性度が大で肉眼的正常肝は高く，表面不平滑肝を最高として硬変肝となつて下り，肝癌に至つて活性度は再び正常肝に近づく，アルカリ性側の極大値をみると肝病変の亢進に伴つて階段的に活性度を増し，肝癌では顕著に高い値を示した。DNA脱アミノ酵素はpH曲線中にさきと同様に二つの極大値 (pH 6 および 9) をみた。酸性側の極大値においては硬変肝の示す活性度が最も高く，肝癌はこれに次ぐが正常肝よりなお著しく高い。アルカリ性側の極大値においては，正常肝より病変肝は著明に活性度を示すが病変肝相互の差異は明らかでない。

核酸の如き酵素作用をこうむる個処の多い複雑な物質を基質として選んだため，肝の水均質液によつて核酸分子に相異なつた酵素作用が同時にあるいは連鎖的におこりうる。われわれはそのうち脱アミノ作用のみに注目して活性度を比較検討したのであるから，なお多くの問題が残されているはずである。われわれは本実験において組織増殖の問題に直結している物質である核酸を採り上げて基質とし，核酸脱アミノ作用の著差が肝の組織の悪性変化に伴つておこることを認めた。要約すればRNAおよびDNA脱アミノ酵素の活性度は病変肝において常に対照より亢進していることである。

(文部省科学研究費による)

[GANN, Vol. 45; March, 1954]

LIVER DESAMINASE ACTION ON HALOGENATED FATTY ACID AMIDES IN RATS FED ON HEPATIC CARCINOGENS

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Few reports have hitherto been written about the enzymatic hydrolysis of halogenated fatty acid amides by an animal tissues extract. A negative result of activity of hydrolysis of chloroacetamide and α-chloropropionamide has been found in liver and kidney of rats by Errera and Greenstein, and in this connection they have briefly described in their paper on desaminase activity on homologous amino acid amides.

First of all we tried to determine the activity of liver desaminase using α-bromo-i-caproamide as substrate and found that it is available for our investigation of desaminase activity. Then some homologous bromo- and chloro- fatty acid amides were synthesized and brought in to enzymatic studies on liver of rats fed on carcinogens, p-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorene (AAF).

METHODS

Experimental animals.—DAB rats. Albino rats weighing about 120 g were placed on rice feed containing 0.06 per cent DAB for 120–150 days continuously until they were killed. The large part of them were replaced to normal diet after the above mentioned period of DAB feeding for 30–40 days successively.

AAF rats. The rats fed on rice containing 0.03 per cent AAF (Eastman Kodak Company) for 150–180 days and they were then placed on normal diet for 90–130 days.

Normal rats. Untreated rats of similar age were fed on normal diet (rice grains).

Enzyme preparation.—The hepatic tissues were removed from freshly sacrificed rats and weighed and homogenized. To the tissue paste distilled water was added adjusting the total volume to 5 and 20 times dilution of original fresh tissue.

Substrates. Bromo- and chloro- fatty acid amides were synthesized in our laboratory, as listed in Table 1 together with their melting point. The analytical data of contained nitrogen were determined by micro-Kjeldahl method and halogen by micro-Carius method.

α-Bromo-fatty acid amides. At first α-bromo-fatty acid bromide was synthe-

Table 1

Homologous bromo- and chloro-fatty acid amides	m. p. (C)	N %	Halogen %
		Obs. value (Calc. value)	Obs. value (Calc. value)
Bromoacetamide	89–90°	10.2 (10.2)	57.1 (57.9)
α-Bromopropionamide	122–123°	9.01 (9.2)	52.2 (52.6)
α-Bromo-n-butyramide	111°	8.2 (8.4)	47.7 (48.1)
α-Bromo-i-butyramide	147°	8.4 (8.4)	46.5 (48.1)
α-Bromo-n-valeramide	83–84°	7.6 (7.8)	43.9 (44.4)
α-Bromo-i-valeramide	130–131°	7.9 (7.8)	43.9 (44.4)
α-Bromo-n-caproamide	59°	7.0 (7.2)	40.1 (41.2)
α-Bromo-i-caproamide	96–97°	7.1 (7.2)	39.3 (41.2)
Chloroacetamide	118°	15.1 (15.0)	36.5 (37.9)
α-Chloropropionamide	79°	13.0 (13.0)	32.0 (33.0)
α-Chloro-n-butyramide	77.5–78°	11.4 (11.5)	28.4 (29.2)

sized, starting with the fatty acid by the action of bromine and red phosphorus (Hell-Volhard-Zelinsky method). Then the product was dissolved in chloroform and treated with aqueous ammonia under cooling to convert it into amide.

α-Chloro-fatty acid amides. α-Chloro-fatty acid was first prepared through chlorination under exposure to sunlight and was esterified. α-Chloro-fatty acid ester thus obtained was converted by interaction with ammonia into amides.

Some synthesized halogenated fatty acid amides had an asymmetric carbon atom in the molecule and they were racemic.

The constitutents of digestion mixtures and the conditions of incubation are represented in Table 2, where three substances listed in Table 1 are omitted for the following reasons: Two of them, chloroacetamide and α-chloropropionamide, were decomposed markedly during incubation even in the absence of enzyme extract, and the third, bromoacetamide, owing to the lack of sufficient material.

Table 2

Name of chemicals	Amount of substrate		Enzyme preparation		Phosphate buffer solution 0.1 M	H_2O	Time in hrs. for incubation at 38°C
	Mol.	cc	Dilution	cc	cc	cc	
1) α-Bromopropionamide	1/15	5	20 ×	2	3 (pH 8)	0	4

2) α-Bromo-n-butyramide	1/15	5	20 ×	2	3 (pH 8)	0	4
3) α-Bromo-i-butyramide	1/30	10	5 ×	2	3 (pH 8)	0	4
4) α-Bromo-n-valeramide	1/15	5	20 ×	2	3 (pH 8)	0	4
5) α-Bromo-i-valeramide	1/30	10	5 ×	2	3 (pH 9)	0	24
6) α-Bromo-n-caproamide	1/30	10	20 ×	2	3 (pH 8)	0	4
7) α-Bromo-i-caproamide	1/60	10	20 ×	2	4 (pH 9)	4	4
8) α-Chloro-n-butyramide	1/10	5	20 ×	2	3 (pH 9)	0	4

Constituents of digestion mixtures and conditions of incubation are represented in Table 2.

The activity of desaminase was measured by the amount of ammonia evolved from substrate by Folin's method taking in consideration the results of blanks and was calculated for ammonia nitrogen mg per g hepatic tissue in 4 hours digestion at 38°C; in case of α-bromo-i-valeramide 24 hours.

Results

Mean values of 5 to 10 activity measurements of each hepatic tissues are

Chart 9

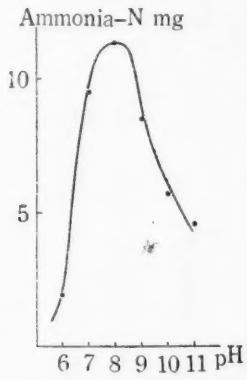
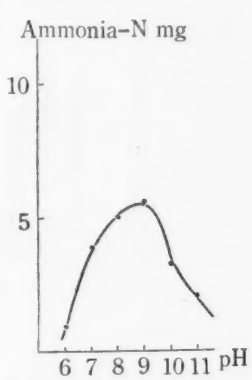


Chart 10



Explanation for Charts 9 and 10

pH-Activity curves of desaminase in normal liver extract by using substrates α-bromo-n-caproamide (Chart 9) and α-bromo-i-caproamide (Chart 10).

Activity was represented ammonia nitrogen mg evolved from substrate per g fresh tissue after 4 hrs. incubation at 38°C.

Optimum pH was shown 8 in the former substrate and 9 in the latter.

Constituents of digestion mixture are in Table 2.

illustrated in Charts 1 to 8. The explanation for them given elsewhere would suffice and further description unnecessary.

The optimum pH of the present enzyme was 8–9, as shown in the pH-activity curves in Charts 9 and 10 by using enzyme extract of normal liver.

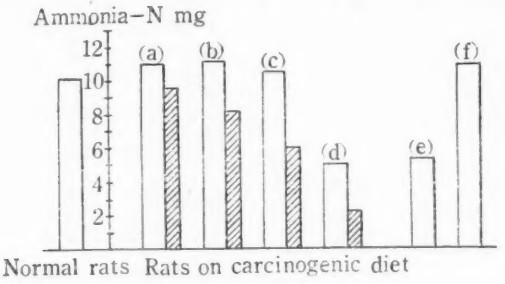


Chart 1 Substrate: α-bromo-propionamide

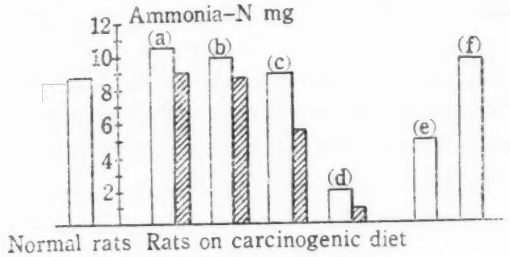


Chart 2 Substrate: α-bromo-n-butyramide

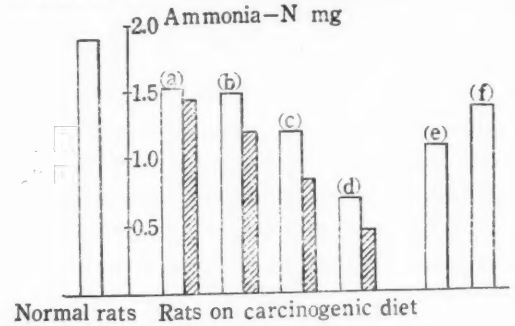


Chart 3 Substrate: α-bromo-i-butyramide

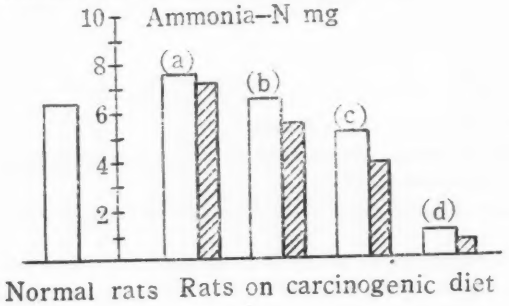


Chart 4 Substrate: α-bromo-n-valeramide

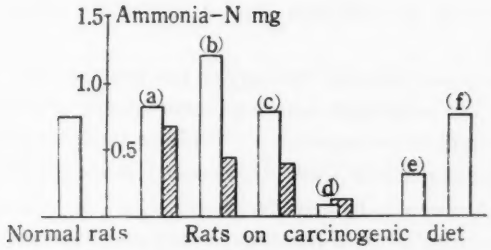


Chart 5 Substrate: α-bromo-i-valeramide

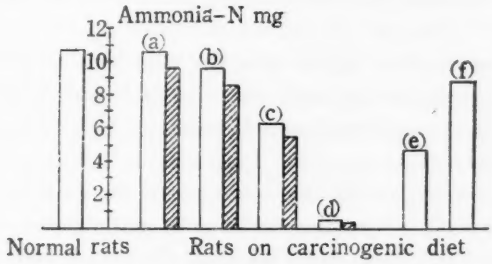


Chart 6 Substrate: α-bromo-n-caproamide

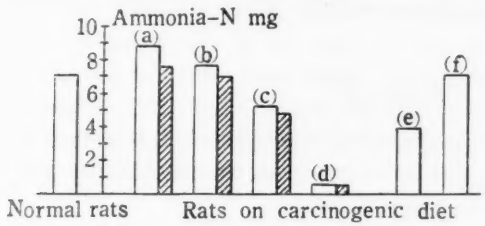


Chart 7 Substrate: α-bromo-i-caproamide

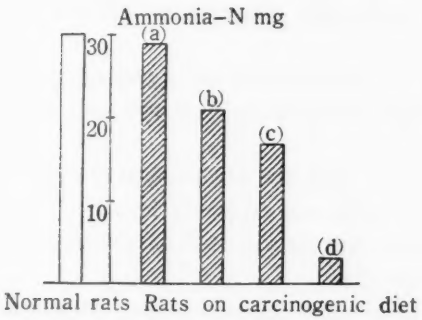


Chart 8 Substrate: α-chloro-n-butyramide

Discussion

Hepatic tissues displayed a specific amidase activity when homologous bromo- and chloro- fatty acid amides have been employed as substrates.

Chloroacetamide and α-chloropropionamide were not available for present ex-

periments owing to their liberation of ammonia during incubation where no enzyme preparation was present.

All substrates except the above two behaved similary to the enzyme and were suitable for experimental use, and especially α-bromo-n-caproamide, α-bromo-i-caproamide and α-chloro-n-butyramide were convenient. It seems that compounds of straight-chain and rich in carbon atoms were decomposed more readily by enzymatic action than of iso and lower fatty acid derivatives.

Normal liver had a high enzyme activity in question; when the liver suffered more or less from pathological changes, however, the activity was lowered usually according their grades of changes.

A slightly affected liver showed often higher activity than that of normal liver especially when a certain substrate was used (cf. Charts 1, 2, 4, 5 and 7).

When the liver turned cancerous the activity dropped extremely low, regardless of the difference in the carcinogen used.

The pathological but non-cancerous liver of rats continuously fed on DAB diet until the measurement has been carried out the desaminase activity was lower than that of rats later discontinued from DAB-feeding. This fact has close resemblance to the case of asparaginase activity which we have reported elsewhere.

Summary

1) The enzymatic hydrolysis of homologous bromo- and chloro- fatty acid amides in the liver of rats fed on hepatic carcinogen, p-dimethylaminoazobenzene or 2-acetylaminofluorene, has been investigated.

2) Aqueous homogenate of normal liver had the marked activity to evolve ammonia from the obove mentioned substrates. Its optimum pH was situated in the range of pH 8 to 9.

3) The phathological but non-cancerous liver showed fairly good activity, when the rats have been removed from their carcinogenic diet and were placed on normal diet for many weeks.

4) Usually the activity fell keeping step with the development of liver lesions and in case of hepatoma nodulus the activity was extremely low.

5) The pathological but non-cancerous liver of rats fed on p-dimethylamino-azobenzene continuously showed low activity.

6) The substrates tested seem to have some characteristics. Aliphatic compounds of straight-chained and rich in carbon atoms were decomposed easier by liver extract than iso and lower fatty acid derivatives.

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Explanation For Charts 1—8

Desaminase activities of hepatic tissues for eight kinds of substrate are represented in Charts 1—8 respectively in comparison with that of normal liver.

Unshaded areas (a), (b), (c), (d) and (f) represent the activities of hepatic tissues of rats fed on DAB diet for 120–150 days and were interrupted for 30–40 days. (a), (b) and (c) show pathological but non-cancerous liver, namely, macroscopically normal liver (a), liver of uneven surface (b) and cirrhotic liver (c); (d) indicate the activity of hepatoma nodulus induced by DAB. (f) is the mean value of (a), (b) and (c), and is illustrated compared with (e). (e) represent the activity of the pathological but non-cancerous liver of rats fed on DAB diet for 120–150 days without interruption until the measurement has been carried out.

Shaded areas represent the activities of the hepatic tissues of rats fed on AAF diet for 150–180 days and later discontinued and kept on the normal diet for 90–130 days. Shaded areas (a), (b) and (c) are the pathological but non-cancerous livers and classified after their hepatic lesions, according to the idea of Wilson et al., namely, liver of no hyperplasia (a), liver of nodular hyperplasia (b), and liver of marked nodular hyperplasia (c). Shaded area (d) shows the activity of hepatoma nodulus induced by AAF.

要　旨

肝癌生成物質投与ダイコクネズミ肝のハロゲン化脂肪酸アミドの脱アミノ酵素について

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われわれは発癌過程における肝酵素作用の変調の追究をつづけている。基質として用いる化学的物質のC—N結合を開く酵素に属するアミダーゼを調べているが，ここにハロゲン化低級脂肪酸アミドの11種を合成しうち8種について得た結果を報告する。

ダイコクネズミに肝癌生成物質バターエロー，アセチールアミノフルオレンをそれぞれ経口投与し一定期間後その肝性組織を採り，水均質液を作り酵素液とした。これに基質としてハロゲン化脂肪酸アミドの水溶液を加え，燐酸緩衝液とともに孵卵器中に保存，一定時間内に脱アミノ化されて生じたアンモニアをフォリン氏法によつて定量し，この種の酵素の活性度とした。

正常肝は上記基質をよく分解する。発癌剤投与中の動物は活性度は低いが，投与を中絶して正常食に戻したネズミの肝では，病変肝でも活性度はかなり高く，軽度の病変では正常よりかえつて高いことがある。しかしすでに肝癌になつている部の活性度は極わめて低い。なお基質とした同族列はみな同一傾向の結果を示した。しかも既報のアスパラギナーゼの場合と酷似している。

この種のハロゲン化脂肪酸アミドの脱アミノ酵素が動物組織中に存在することは文献に見当らない。使用した基質のうち炭素原子数の多いノルマル脂肪酸誘導体はイソや，より低級脂肪酸誘導体より顕著にネズミ肝均質液により脱アミノ化される傾向がみられる。

(厚生科学研究費による)

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DIALYZABLE FORM OF TOXOHORMONE. A SIXTH STUDY ON TOXOHORMONE, A CHARACTERISTIC TOXIC SUBSTANCE PRODUCED BY CANCER TISSUE.

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INTRODUCTION

Ever since our first separation of the characteristic toxic substance produced by cancer tissue (toxohormone), which causes the well known depression of liver catalase activity in cancer patients and cancer bearing animals (1), it has been our main concern to elucidate the properties of that substance. In our early experiments we only showed that the substance is water soluble, non-heat coagulable, heat stable, and can be concentrated by means of successive precipitations with alcohol and other protein precipitants (2). The most potent preparation we have been able to obtain was active in 5 mg doses for mice (3). Greenfield and Meister (4) then published the results of their experiments, confirming and greatly extending our original observations.

One of the most significant new facts brought out by Greenfield and Meister was that the toxohormone concentrate, obtained by them by precipitation with alcohol, retained its activity even after it was hydrolyzed with hydrochloric acid, though they made no comment on the relation of this remarkable fact to the original active substance, which they stated is non-dialyzable. Later, Hargreaves and Deutsch (5) reported a dialyzable tumor substance which is capable of depressing, under certain experimental conditions, the catalase activity *in vitro*. The catalase depressing substance (toxohormone), as originally separated by us or by Greenfield and Meister, is active only *in vivo*, and is devoid of *in vitro* effect on catalase. This fact was reiterated more recently by Greenfield and Price (6).

Experiments we report in the present paper show that tumor extract contains a small amount of dialyzable substance which depresses liver catalase activity when injected into normal mice, and that the non-dialyzable toxohormone concentrate, when digested with papain or pepsin, yields a dialyzable substance which is potent *in vivo* exactly as the active substance occurring in the dialysate of fresh tumor extract. These facts definitely point to the conclusion that toxohormone occurs in two forms, one non-dialyzable and the other dialyzable, and that the latter may be derived from the former by a simple biochemical

process. This conclusion introduces a new concept for the fundamental properties of toxohormone.

Material and Methods

The tumor used as the source of toxohormone in the following experiments was the NF strain of transplantable mouse sarcoma, which has been maintained in our laboratory since 1948, now for more than 120 transplantation generations.

The *in vivo* activity of various tumor fractions was tested as usual by injecting them intraperitoneally into normal mice of hybrid albino strain, weighing 15~20 g, and by determining the liver catalase activity 24 hours after the injection. For the catalase test gas-volumetric method was used as before, and the details of our previous procedures were most meticulously followed: 0.1 g of liver tissue from a mouse killed by exsanguination thoroughly mashed and extracted with 10 cc of phosphate buffer of pH 7, 0.3 cc of the centrifuged extract diluted with 0.7 cc of the same buffer solution, and the mixture allowed to react with 10 cc of 3 per cent hydrogen peroxide solution for 5 minutes at room temperature. The volume of oxygen was reculculated to that at the standard condition.

Our experience with the above procedure of the catalase determination for the past ten years or more, using literally hundreds of mice, provided sufficient empirical grounds for accepting any figure less than 4 cc oxygen as being beyond the lowest limit of the normal. The average liver catalase activity in normal mice is represented by about 6~7 cc oxygen.

The results of the experiments reported in the following lines are judged according to these standards.

Toxohormone Activity of Tumor Dialysate

The first point established in the present series of experiments was the existence of the liver catalase depressing factor in the dialysate of extract prepared from fresh tumor tissue.

Experiment 1. Freshly removed NF sarcoma tissue was thoroughly mashed and extracted with 10 cc per 1 g ratio of distilled water for about 1 hour with vigorous shaking. The extract was separated first by filtration through layers of gauze and then by centrifugation, and was dialyzed through animal membrane against the equal volume of distilled water at the temperature of a few degrees above 0°C for 3 to 5 days. (Re-dialysis under similar conditions proved to yield very little additional dialysate and was therefore not generally carried out).

The almost colorless dialysate was either evoporated to dryness, or was concentrated to a suitable volume and twice the volume of absolute alcohol added, thus separating into alcohol precipitate and filtrate.

Yields varied from batch to batch, but on an average the total dialysate amounted to about 10 mg per 1 g of fresh tumor tissue, and the ratio of alcohol precipitate and filtrate was something like 1 : 30. The total dialysate and alcohol filtrate were somewhat sticky, hygroscopic matter of yellowish brown color; alcohol precipitate greyish white powder.

These materials were injected into normal mice in varying doses and the effect on the liver catalase activity determined according to the method already fully described. Table 1 gives the results of these determinations.

Table 1

Material tested	Amount mg	Mouse No.	Liver catalase activity Oxygen cc
Whole dialysate	100	1	3.0
		2	4.8
	50	3	2.8
		4	4.3
Alcohol precipitate	100	5	Died overnight
	50	6	5.5
		7	5.6
Alcohol filtrate	50	8	3.5
		9	3.7
		10	3.8
		11	4.5
	25	12	3.9
		13	4.1

According to our empirical standards, the above results may be sufficient to demonstrate the existence of the catalase depressing substance in the fresh tumor dialysate.

In dividing the dialysate into alcohol precipitate and filtrate in the above experiment we had in mind the well established fact that toxohormone can be separated from tumor extract by precipitation with alcohol. It was, therefore, somewhat to our surprise that the catalase depressing action of the dialysate was concentrated in alcohol filtrate, and not in the precipitate. This result should perhaps have been expected, however, since the precipitability with alcohol would depend on the states in which toxohormone may occur in solution.

Experiment 2. The above experiment was repeated using dried, instead of fresh, tumor tissue as the starting material.

NF sarcoma tissue, previously dried over boiling water bath and stored for

some time in a desiccator over calcium chloride, was pulverized and extracted with distilled water in amount of 10 cc per 1 g in boiling water bath for 1 hour. Extract separated by centrifugation was dialyzed through animal membrane against the equal volume of distilled water at the temperature of a few degrees above 0°C for 3~5 days. The dialysate obtained was slightly tinged with brownish yellow. It was concentrated to a suitable valume, a small amount of coagula formed was centrifuged off, and to the clear concentrated solution was added twice the volume of absolute alcohol. On an average 2 mg per 1 g original dried tissue of precipitate and 30 mg of filtrate were obtained, the former greyish white powder, the latter brownish yellow, sticky, hygroscopic matter.

The results of testing the liver catalase depressing effect of these materials were as follows (Table 2):

Table 2

Material tested	Amount mg	Mouse No.	Liver catalase activity Oxygen cc
Alcohol precipitate	100	1	Died overnight
	50	2	6.7
		3	8.6
	25	4	6.0
		5	8.4
Alcohol filtrate	100	6	Died overnight
		7	3.9
	50	8	3.0
		9	3.6
		10	4.0
		11	5.3

Experiment 3. In this experiment we made some attempts at the purification of toxohormone in dialysate. Although the results were unsatisfactory, they are quoted heres imply as additional data for the dialysable form of toxohormone.

1 g of pooled alcohol filtrates, samples left over from the two preceding experiments, was dissolved in 10 cc of distilled water, and mixed with the equal volume of saturated aqueous solution of picric acid, heated over boiling water bath until evaporated almost to dryness, cooled and diluted after cooling to the original volume with distilled water, producing yellow precipitate (amorphous picrate), which, when extracted with acetone to remove picric acid, yielded about 20 mg of residual substance.

The clear filtrate of brown color was evaporated to a small volume and was kept in a refregerator at a few degrees above 0°C overnight. Crystalline picrate

separated out, from which close to 5 mg of residual substance was regenerated after decomposing the crystals and removing picric acid by means of acetone.

The mother liquor from which the crystals of picrate were removed was dark drown syrup, amounting to 680 mg.

Test on normal mice showed that the active substance has not been obtained as picrate, either amorphous or crystalline. The mother liquor, on the contrary, was found to carry the substance. See Table 3.

Table 3

Material tested	Amount mg	Mouse No.	Liver catalase activity Oxygen cc
Amorphous picrate	20	1	5.7
Crystallin picrate	5	2	6.1
Mother liquor	50	3	3.4
		4	3.8
		5	3.8
		6	4.0

Enzymic Conversion of Non-dialyzable Toxohormone into Dialyzable Form

Having thus established the existence of dialyzable form of toxohormone, the next problem was to determine, if possible, the connection between this form and the well known non-dialyzable form. In the experiments to follow, we found that the concentrate of non-dialyzable toxohormone yields the dialyzable form when subjected to enzymic digestion, demonstrating that the dialyzable form may be derived from the non-dialyzable one by a simple splitting process.

Experiment 4. Papain was used in this experiment to digest the toxohormone concentrate of non-dialyzable form.

Fresh NF sarcoma extract, subjected to dialysis in Experiment 1 described in the preceding section, i. e., the non-dialyzable fraction of the extract, was treated with twice the volume of absolute alcohol and the precipitate obtained was used as the source of the non-dialyzable form of toxohormone.

3 g of this material and 0.5 g of papain preparation (Merck) were dissolved in 150 cc of distilled water, and the mixture (pH 6) was incubated at 37°C for 4 days under toluol. The digest was heated at 100°C, coagula formed was centrifuged off, and the clear fluid was dialyzed against 200 cc of distilled water for 5 days at the temperature a few degrees above 0°C. Dialysate obtained was concentrated to a suitable volume and was precipitated by adding two volumes of absolute alcohol. Yields: 120 mg of precipitate and 400 mg of filtrate, the former

greyish white powder and the latter hygroscopic, orange yellow matter.

The toxohormone activity of the precipitate and filtrate was tested with the following results (Table 4):

Table 4

Material tested	Amount mg	Mouse No.	Liver catalase activity Oxygen cc
Precipitate	60	1	5.4
	30	2	5.9
		3	5.6
Filtrate	50	4	2.7
		5	3.3
	25	6	3.6
		7	3.9
		8	4.3

Experiment 5. The above experiment was repeated, using pepsin.

2 g of alcohol precipitate of non-dialyzable toxohormone concentrate and 0.5 g of pepsin preparation (Baltimore Biological Laboratory, Inc.) were dissolved in 200 cc of N/10 HCl solution, and digested under toluol at 37°C for 4 days. Digest was then heated at 100°C, neutralized with NaOH, and put to dialysis through animal membrane against 200 cc of distilled water for 5 days at 2~3°C. Dialysate was concentrated to a small volume and precipitated with twice the volume of absolute alcohol as before. 100 mg of precipitate and over 700 mg of filtrate were obtained. The toxohormone activity of these materials was as follows (Table 5):

Table 5

Material tested	Amount mg	Mouse No.	Liver catalase activity Oxygen cc
Precipitate	50	1	5.3
	25	2	5.6
		3	6.1
Filtrate	100	4	4.0
	50	5	3.6
		6	3.8
	25	7	3.9
		8	5.1

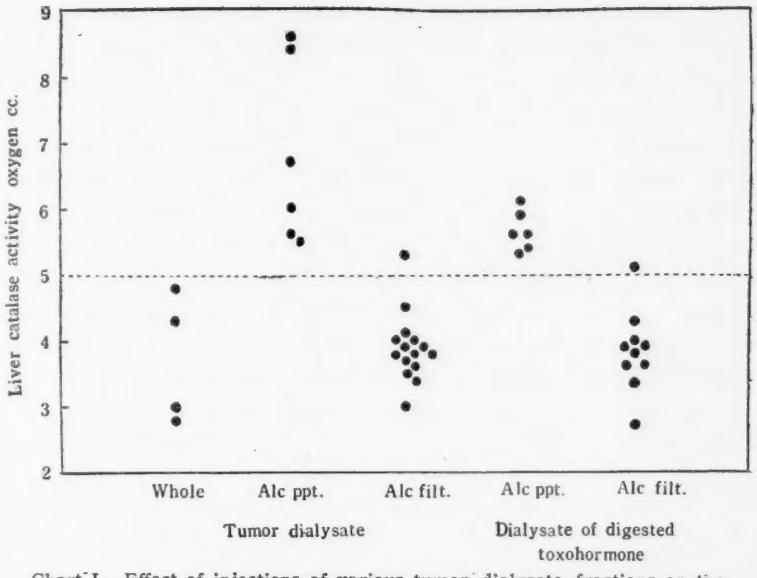

Chart I. Effect of injections of various tumor dialysate fractions on the liver catalase activity of mice.
(Composit representation of Experiments 1—5)

Discussion

That toxohormone comes in dialyzable and non-dialyzable forms, and that the former may be derived from the latter by a simple enzymic dissociation seems to provide adequate grounds for the assumption that the elementary form of this unique toxic product of cancer tissue may be of relatively small molecule.

It might occur in cancer tissue mostly either as aggregates of such elementary forms or as conjugates with some other substance, and the toxohormone originally separated by us and by Greenfield and Meister by means of precipitation with alcohol may represent this aggregate or conjugate form. The fact that toxohormone is precipitated by alcohol at a very wide range of concentrations, without showing a sharp optimum concentration, is in keeping with the idea that the aggregates may be of varying sizes, or that the conjugate may be formed with divergent proportions of another substance or substances. Attempts at the isoelectric precipitation of toxohormone never yielded a satisfactory separation, and these experiences may also be taken to speak for the same idea.

Greenfield and Meister (4) previously reported a significant fact that toxohormone concentrate retains its activity even after being subjected to acid hydrolysis. In the light of our present findings, this may mean that the aggregate or conjugate form of toxohormone, constituting the active principle

of the non-dialyzable concentrate, is converted to the elementary form, which is not completely decomposed even by the drastic hydrolytic process, and at least enough of it remains to produce the depression of liver catalase when injected into normal mice.

At this stage of our investigation it is yet premature to consider the relation of the dialyzable form of toxohormone to the "Kochsaft" factor of Hargreaves and Deutsch (5). "Kochsaft" factor is said to be capable of inhibiting catalase *in vitro* under certain specified experimental conditions. It is released from tumor tissue by heating at 100°C, soluble in 80 per cent alcohol and dialyzable, and it effects a marked change in the absorption spectrum of catalase. In a preliminary way we tested the effect *in vitro* on catalase of our active dialysate fractions, adhering closely to the experimental conditions prescribed by Hargreaves and Deutsch, but the results were erratic and inconclusive.

There is another point which render difficult the interpretation of "Kochsaft" factor in the light of our dialyzable form of toxohormone, and that is that our substance is readily extractable from fresh tumor tissue, while "Kochsaft" factor is claimed to be released only after heating the tissue. We tried to envisage the situation in the term of "free" and "bound" forms of toxohormone, and, under this hypothetical scheme, we suspected that the "free" form might prove non-dialyzable and "bound" form dialyzable, the latter corresponding to "Kochsaft" factor. Our experimental results, which need not be given in detail here, only showed that tumor tissue, which was repeatedly extracted so that it no longer yields much toxohormone, can, if boiled, again yield an increased amount of the active substance. The renewed yield of toxohormone from boiled tissue, however, may mean nothing more than the usual better extractability of any substance under heating.

The correct interpretation and proper evaluation of Hargreaves and Deutsch's observations must await further investigation.

Summary

Experiments were described demonstrating that tumor extract contains a dialyzable substance which, when injected into normal mice, markedly depresses the liver catalase activity, and that enzymic digestion of the non-dialyzable toxohormone concentrate readily produces the dialyzable substance with similar activity.

Possibility was suggested that the dialyzable factor may represent the elementary form of toxohormone, which may occur in cancer tissue mostly as aggregates of such forms or as conjugates with some other substance.

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要　旨

トキソホルモンの可透析型

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腫瘍組織の水浸出液を透析して，透析物中にトキソホルモン作用を有する物質が存在すること，および従来得られた非透析性のトキソホルモン濃縮物を酵素的に分解すると，同様の可透析性トキソホルモン作用を有する物質が得られることを見出した。

可透析性のものがトキソホルモンの基本型であり，非透析性のものはこの基本型の集合した，あるいは他の物質と結合した型であるまいかと考えられる。Greenfield-Meister の非透析性トキソホルモン濃縮物を塩酸水解してもなお効力を失わないという成績は，これによつて説明出来る。

(文部省科学研究費及び厚生省厚生科学研究費による)

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BIOSYNTHESIS OF TOXOHORMONE. A SEVENTH STUDY ON TOXOHORMONE, THE CHARACTERISTIC TOXIC SUBSTANCE PRODUCED BY CANCER TISSUE.

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INTRODUCTION

As regards the chemical nature of the characteristic cancer toxin (toxohormone), which is assayable by its depressing effect on the liver catalase activity, our early experiments indicated that it may be a kind of proteose or polypeptide (1, 2), and the more recent demonstration of the dialyzable form showed that this substance in its elementary form may be of a relatively small molecule (3). In the present experiments, in order to obtain further light on the problem, we considered the possibility of producing toxohormone biosynthetically on the basis of the working hypothesis that the substance in question may be a polypeptide.

If the assumption is true that toxohormone is of the nature of polypeptide, it may be expected to be synthesized by cancer tissue from free amino acids normally occurring in the tissue, but past studies on the free amino acids give no indication as to the kinds of amino acids which may be implicated. However, Greenfield and Meister (4) detected in the hydrolyzate of a toxohormone concentrate the presence of the following amino acids: alanine, glycine, serine, proline, aspartic acid, arginine, valine, threonine, phenylalanine, hydroxyproline, α-aminobutyric acid, isoleucine, tryptophane, lysine, leucine and glutamic acid. The absence of tyrosine and histidine was noteworthy. Okushima (5), who investigated amino acid composition of toxohormone fractions which he prepared according to our earlier methods, also reported the presence of alanine, glycine, serine, proline, aspartic acid, arginine, valine, threonine, lysine, leucine and glutamic acid. The number of possibly relevant amino acids was reduced to eight by Fukuoka and Nakahara (6) by testing the effect of various amino acid mixtures on the toxohormone contents of tumor tissue. They found that a mixture of alanine, proline, aspartic acid, arginine, phenylalanine, lysine, leucine, and glutamic acid, when injected into mice bearing tumors, produced two to three fold increase in the toxohormone activity of the tumor tissue homogenate,

and pointed out that these amino acids, more than others, may be concerned in the synthesis of toxohormone, possibly serving as the building material.

In this connection, the recent discovery of enzymic systems which bring about the *in vitro* synthesis of certain peptide-like bonds with the concomittant breakdown of adenosinetriphosphate, and especially the ATP-dependent biosynthesis of the tripeptide glutathione (γ-glutamylcyteinylglycine) by an enzyme system from liver tissue, cannot fail to be of great interest (7). It was indeed under the influence of these brilliant pieces of work that we have been led to the speculation that biosynthesis of toxohormone may be possible in a somewhat similar manner.

In attempted experiments we were most fortunate to find that a substance highly active as toxohormone can readily be produced *in vitro* by allowing fresh tumor slices to act upon amino acid mixtures in the presence of ATP, and that, apparently, only three amino acids, arginine, phenylalanine and leucine, need to be included in the reaction mixture.

Although these results do not necessarily prove that toxohormone is a polypeptide, they nevertheless offer a definite advantage to future studies by establishing relatively simple experimental conditions under which this unique substance can be produced.

Biosynthesis Experiments

Experiment 1. The reaction mixtures were composed as follows:—

1. Amino acids. 1 mg each of crystalline alanine, proline, aspartic acid, arginine, and phenylalanine, 2 mg each of lysine and leusine, and 4 mg of glutamic acid were used in each mixture. Of these amino acids, phenylalanine and lysine were synthetically prepared dl-forms, and the rest natural l-forms.

Nine different mixtures were prepared; one including all the eight amino acids, eight others excluding in turn only one of the eight, thus to test the possible rôle of each of the eight amino acids.

Each mixture of amino acids was dissolved in 2 cc of distilled water, and the reaction of the solution adjusted to pH 7 with NaOH.

2. ATP. Ba-salt of adenosinetriphosphate, prepared and kindly donated to us by Mr. Kazuo Kuretani of Dr. Saburo Ugami's Laboratory, Scientific Research Institute, Tokyo, was used. It was decomposed and barium removed by sulphuric acid, and neutralized, according to the usual method, and the amount corresponding to the yield from 5 mg Ba-salt was added to each amino acid mixture, which was then steam sterilized.

3. Tumor slices. Necrosis and hemorrhage free tissue of NF strain of transplantable mouse sarcoma, removed immediately after killing the mouse, was cut into thine slices, and three slices amounting to approximately 50 mg were

placed in each mixture. All the manipulations were carried out under the strict surgical asepsis.

The reaction mixtures prepared as described above were then placed in an incubator at 37°C for 24 hours, during which time they were shaken lightly only two or three times.

After the incubation the mixtures were heated to 100°C, the solution containing fine coagula was decanted, discarding the tumor slices. Each solution was then injected into two young adult normal mice intraperitoneally, each mouse receiving one-half of the total solution. The injections produced but very slight and transitory discomfort to the mice.

The liver catalase activity of the mice was determined 24 hours after the injection, strictly according to our customary gas-volumetric method, for which our previous publications may be consulted. It is pertinent only to state that with this method 0.4 cc value of oxygen indicates practically the lowest limit of the activity in normal untreated mice, and that anything less than that value represents unequivocal depression. The average normal is about 6~7 cc oxygen.

The results of this experiment were as follows (Table 1):—

Table 1

Amino acids in reaction mixture	Mouse No.	Liver catalase activity Oxygen cc
8 amino acids	1 2	2.2 3.0
Alanine omitted	3 4	2.0 2.9
Proline omitted	5 6	2.2 2.9
Aspartic acid omitted	7 8	2.9 3.6
Arginine omitted	9 10	4.4 5.7
Phenylalanine omitted	11 12	4.8 5.6
Lysine omitted	13 14	3.6 3.8
Leucine omitted	15 16	3.3 3.7
Glutamic acid omitted	17 18	3.2 3.8
Untreated control	19	7.4

The amount of toxohormone originally contained in the tumor slices, even if all of it had diffused out of the slices, could not possibly account for the marked activity of the reaction mixture noted in the above results, for we know that it takes an injection of something like 600~800 mg of the whole homogenate of this tumor tissue to produce unmistakable depression of the liver catalase activity (Fukuoka and Nakahara (6)). It is well established, also, that injections of mere mixtures of amino acids do not affect the liver catalase activity (Greenfield and Meister (5)), while the effect of adenosinetriphosphate itself is out of the question. The only reasonable conclusion, then, is that there was actually a synthesis of a substance with powerful toxohormone action in the reaction mixture.

It is quite clear in the above results that omitting alanine, proline or aspartic acid had absolutely no detectable effect. These three amino acids may, therefore, be regarded as of no importance in the biosynthetical reaction.

The cases of omitting arginine or phenylalanine, on the contrary, indicated that there may have been practically no synthesis of toxohormone, suggesting that these two amino acids may be indispensable.

Exclusion of lysine, leucine or glutamic acid seemed to have somewhat lowered the toxohormone synthesis and the rôles of these three must be investigated further.

Experiment 2. In view of the results of the preceding experiment we next attempted to clarify the situation by adding leucine, glumatic acid and lysine in various combinations to the reaction mixture containing arginine and phenylalanine, the necessity of which is already apparent.

2 mg each of amino acids were used in this experiment. Each mixture was dissolved in 1 cc of distilled water and the solution neutralized. ATP and tumor slices were added in the same way as in Experiment 1, which was closely followed as to all other experimental procedures.

The results of the experement were as shown in Table 2.

Table 2

Amino acids in reaction mixture	Mouse No.	Liver catalase activity Oxygen cc
Arg+Phe	1	3.5
	2	3.8
	3	5.4
	4	6.2
Arg+Phe+Glu	5	3.6
	6	4.4
Arg+Phe+Leu	7	3.2
	8	3.5
Arg+Phe+Leu+Glu	9	2.6
	10	2.7
Arg+Phe+Leu+Lys	11	2.7
	12	3.1

The above results indicate that although some toxohormone synthesis might be suspected even in the case of arginine+phenylalanine, and arginine+phenylalanine+glutamic acid, the results comparable with the cases of the most powerful toxohormone production were obtained only when arginine, phenylalanine and leucine were included. These three amino acids may therefore be considered as necessary and sufficient for the process. The addition of glutamic acid or lysine was found not to markedly improve the result.

Control Experiments

Having demonstrated that a substance with toxohormone activity can be produced by incubating at 37°C a mixture of at least three amino acids (arginine, phenylalanine and leucine) with fresh tumor tissue slices in the presence of ATP, there remains for us to describe some control experiments.

Experiment 3. This experiment constituted normal tissue control. Three reaction mixtures were prepared in the same manner as before, using the three amino acids and ATP. To one of these was added tumor slices (positive control) and to the remaining two slices of live and skeletal muscle, respectively, from a freshly killed normal mouse were added. Three tissue slices weighing approximately 50 mg were used in all cases. The experimental conditions were otherwise the same as before. The normal tissue controls were prepared in duplicates.

As may be expected neither liver nor muscle slices gave rise to the production of the active substance, which is engendered by tumor slices. See Table 3.

Table 3

Tissue slices used	Mouse No.	Liver catalase activity Oxygen cc
Tumor	1	2.2
	2	3.9
Liver	3	4.9
	4	4.9
	5	5.3
	6	5.9
Muscle	7	4.3
	8	4.7
	9	6.0
	10	6.1

Experiment 4. This experiment was carried out as a sort of blank control. Three mixtures were sat up: First with the three amino acids and ATP as before (positive control), Second with the three amino acid only, and Third with ATP only, sufficient amount of phosphate buffer of pH 7 being added to the last two to equalize the total amount of the three mixtures. Tumor slices were then added equally to each mixture.

Table 4 represents the results of this experiment, which shows that without the presence of both the amino acids and ATP tumor slices fail to produce the active substance.

Table 4

Reaction mixture	Mouse No.	Liver catalase activity Oxygen cc
Tumor slices +amino acids +ATP	1 2	3.4 3.9
Tumor slices +amino acids only	3 4	8.0 8.7
Tumor slices +ATP only	5 6	5.5 6.8
Untreated control	7 8 9	5.4 6.2 8.8

These control experiments showed: (1) the inability of normal tissue slices to produce the toxohormone-active substance, and (2) the absolute necessity of both the amino acids and ATP for the biosynthetical processes. The "positive controls" in these experiments, moreover, adequately reaffirmed the production of the active substance by the reaction of tumor slices and the amino acids in the presence of ATP.

Chart 1. Effect of various combinations of amino acids on the biosynthesis of toxohormone

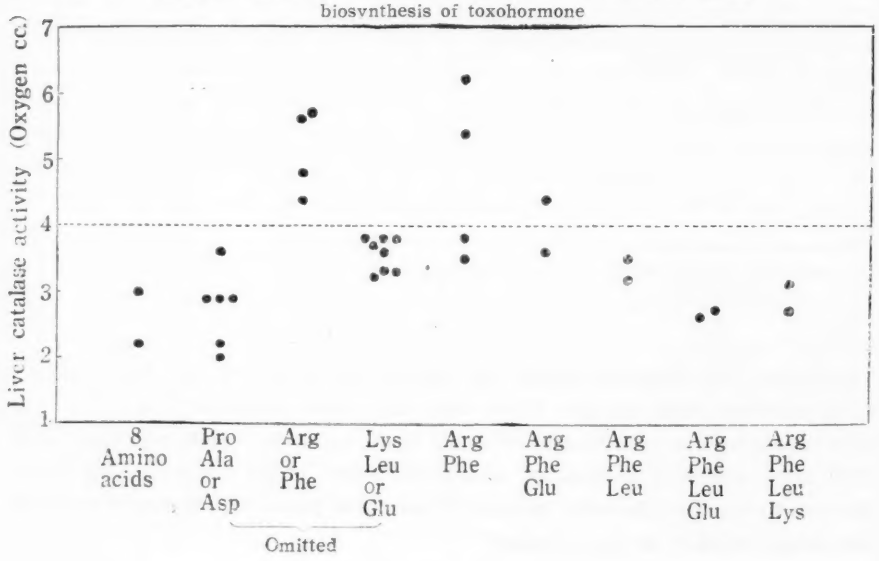
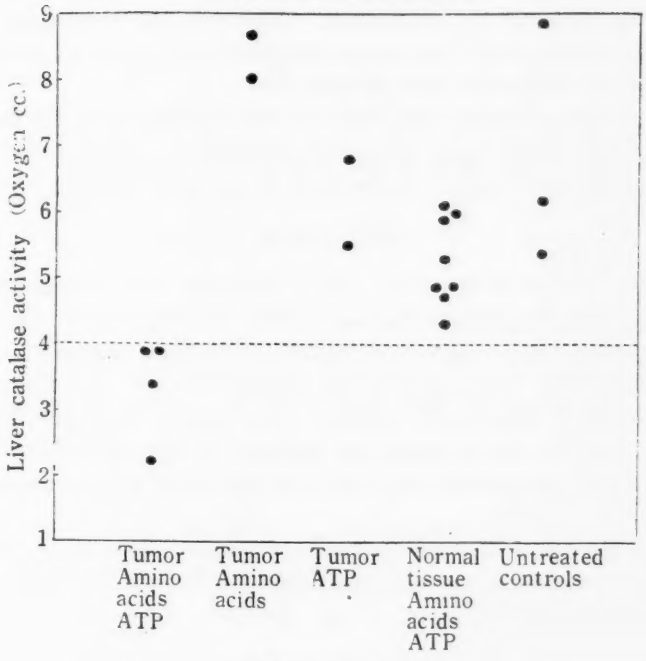


Chart 2. Control experiments



Discussion

In the interpretation of the results of the biosynthesis experiments described in this paper it seems important to take into account what little free amino acids that may be carried into the reaction mixture by the tumor slices. The experimental results at first glance might be taken to mean that even with only two amino acids (arginine and phenylalanine) a certain minimum amount of toxohormone is produced, which can only be accounted for by the amino acids from the tumor slice.

It would seem more than likely that the traces of amino acids supplied by the tumor slices do take part in the biosynthetical reaction. If so, since the amount and kinds of free amino acids contributed by the tumor slices could very well vary depending on individual tumors, it might affect the minimum number of amino acids that must be added to the reaction mixture in order to insure the synthesis of the active substance. Our present contention that arginine, phenylalanine and leucine may be necessary and sufficient is based on experiments with one type of tumors, and it may not strictly apply to the cases of other tumors.

It is possible that the active substance thus produced biosynthetically may be a condensation product of the amino acids and so actually be a polypeptide, but

this point cannot be established without first isolating the substance in a pure state and determining its composition. However, the mechanism involved might be more complicated than a mere condensation of amino acids, and further studies alone can settle this moot question.

We may not fail to mention, also, that the chemical identity of this biosynthetic product and the toxohormone occurring naturally in cancer tissue cannot be absolutely established without the isolation of the pure substances.

Conclusions

We have found that a substance with a powerful toxohormone activity, as assayable by the liver catalase depressing effect on normal mice, can be readily produced *in vitro* when a mixture of certain amino acids is allowed to be acted upon by fresh tumor tissue slices in the presence of ATP. The inclusion in the reaction mixture of three specific amino acids, namely, arginine, phenylalanine and leucine, seems to be necessary and sufficient for this biosynthesis, although the possibility of other amino acids participating in the process cannot be ruled out.

The present paper is of the nature of a preliminary report, and further studies are required to determine the optimum conditions for the maximum production of the active substance *in vitro*.

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要　旨

トキソホルモンの生合成

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トキソホルモンは一種のポリペプタイドであろうという作業仮説から，最近の組織酵素系——ATP によるペプタイドの生合成の研究に倣い，アミノ酸と ATP とを用い腫瘍組織スライスによる試験管内トキソホルモン合成を試み，簡単な要約のもとにトキソホルモンとして強力な作用を有する物質が反応液中にできることを見出した。

この生合成に最少限必要なアミノ酸は，アルギニン，フェニルアラニンおよびロイシンの三種であるが，他のアミノ酸もある程度関与している可能性は否定できない。また，生合成の最適実験要約は将来の研究によつて決定されなければならない。

(文部省科学研究費による)

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